J. Margaret Pittman

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MANUAL

FOR THE

MICROSCOPICAL DIAGNOSIS OF MALARIA IN MAN

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MICROSCOPICAL DIAGNOSIS OF MALARIA IN MAN

By

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FOREWORD

This manual begins with a description of the morphology and life history of the parasites of the different species of malaria, a description which is clear and thorough and should be useful to both the beginner in the subject and to one who may wish a concise review. The author uses throughout the terminology recommended by the Sub-Committee of the Health Organization of the League of Nations.

The bulk of the manual consists of a treatment of the microscopical diagnosis of malaria in man. In this the author makes use of her own wide experience in the subject. Of especial usefulness are the plates showing the appearance of parasites in the thick film. The manual describes thoroughly the technique of the thick and thin films, and not only guides to the straight road leading to a correct diagnosis but also points out the various pitfalls awaiting the unwary microscopist.

This work is of especial use at this time when the expansion of the area of activity of our Army and Navy has brought many persons into malarious regions. I regard this publication as a clear, thorough, and timely treatment of an important subject.

M. A. Barber.

Memphis, September 1941.

PREFACE

This manual is designed to aid the student in the study of malaria and the microscopist in the diagnosis of the disease. It can be a help to the field worker who makes blood surveys for malaria and to the physician who takes blood smears from his patients.

It is hoped that it will prompt the exercise of greater care in the making of blood films sent to laboratories for examination, since well-made films are the basic step toward reliable diagnosis and the best efforts of a qualified technician may be frustrated by the poor condition of the material with which he has to work. It is earnestly desired that it will promote greater precision in microscopic diagnosis of malaria by fostering the use of the thick film by more persons, particularly in small laboratories throughout the South. Many of the large State laboratories already employ the method.

"Because of its frequent fatal termination falciparum infection should be approached with more concern than vivax or malariae infection, and for this purpose correct identification of the species of parasite involved is exceedingly important" (*1*).

Little of this material is original but it represents an accumulation of knowledge gained through work in malaria diagnosis over a period of 14 years.

The terminology in this publication follows exactly that by a Sub-Committee of the League of Nations' Health Organization Malaria Committee (*2*). In a number of places terms in more common use in the United States are indicated in parentheses after the accepted terms.

AIMEE WILCOX.

SEPTEMBER 1941.

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MANUAL FOR THE MICROSCOPICAL DIAGNOSIS OF MALARIA IN MAN

I. THE LIFE HISTORY OF PLASMODIA

Introduction

At present all authorities accept three distinct species of the *Plasmodia* causing malaria in man. These are *Plasmodium vivax*, the cause of benign tertian (tertian) malaria; *P. malariae*, the cause of quartan malaria; and *P. falciparum*, the cause of aestivo-autumnal (malignant tertian, subtertian) malaria. All three of these species are found in the United States, particularly in the southern States, though cases of malaria are found as far north as Wisconsin, Minnesota, and Michigan and as far west as Idaho, Oregon, and California. A fourth species, *P. ovale*, has been recognized and accepted by most authorities, but this species has never been found in persons living in the United States.

The general course of the life history of the malaria parasite is the same for all species. There are two cycles: (1) the sexual, (sporogony) in certain species of anopheline mosquitoes, and (2) the asexual, (schizogony) in man.

Life Cycles

Sexual cycle (in the mosquito).

If a person bitten by a female anopheline mosquito is an infective carrier of malaria, that is, if he has within his blood the necessary number of mature sexual forms (gametocytes), the mosquito will become infected (*3*). When the blood, containing the malaria parasites, is taken into the mosquito's stomach, all the asexual forms and immature gametocytes are digested, but many of the mature sexual forms (called in man the microgametocyte in case of the male, and macrogametocyte in case of the female) not only survive but change form and continue their development. By movements of contraction and expansion they rupture the red blood cells which contain them and escape into the stomach cavity of the mosquito. The microgametocyte throws out from 4 to 8 flagellum-like structures called microgametes, which break away from the microgametocyte, leaving a residual body in which the pigment granules remain. Meanwhile, the macrogametocyte undergoes a change. There is an elimination of part of the chromatin in one or two masses, sometimes called "polar bodies."

This stage of the female parasite is called the macrogamete and it is now ready for fertilization, which takes place when one of the free

microgametes comes in contact with a macrogamete, penetrates it, and the nuclei of the two become fused. This fertilized parasite is called the *zygote*. This soon elongates and becomes motile and is then called the *oökinete*. The oökinete forces its way into the wall of the mosquito's stomach and comes to rest between the outer membrance and the inner epithelial cells. Here it contracts to a small round body, becomes surrounded by a kind of cyst wall and is called an *oöcyst*. It has a single mass of chromatin and a mass of cytoplasm containing the pigment of the original macrogametocyte. The oöcyst grows enormously; the nucleus multiplies by repeated divisions; the cytoplasm develops vacuoles, forming a sponge-like arrangement of interconnecting protoplasmic masses. When vacuolation is complete the nuclei arrange themselves near the surface of the cytoplasm. Above each nucleus a finger-like process of cytoplasm protrudes carrying the nucleus with it, elongating, and finally breaking away as an individual from the unused cytoplasmic mass. Each such individual is called a *sporozoite*. The oöcyst when mature bursts and liberates the sporozoites, which number from several hundred to several thousand per oöcyst, into the body cavity of the mosquito. The sporozoites are thin, pointed at each end, have a nucleus, and are very motile. They migrate to practically all the organs of the mosquito's body and many get into the salivary glands where they lose their motility. When a mosquito bites a man, these sporozoites are injected with the saliva, which is emitted at the time of biting, into the skin, and thus begins the asexual cycle in man.

Asexual cycle (in man).

Explanation of terms.—In this article the term "trophozoite" is used to include the asexual stages from the young form (ring) through the stage where the parasite has completed its vegetative growth and stopped taking nourishment from the cell but, as yet, has an undivided chromatin mass. The rings and young forms are called small trophozoites and the older, ameboid and more solid forms are termed, according to their age, growing and large trophozoites. "Schizont" (presegmenting schizont) is used to denote the asexual stages of the parasite in which the chromatin shows evidence of schizogonic division. There may be such forms with two, four, or more chromatin masses, but the merozoites are not yet differentiated. "Mature schizont" (segmented schizont) is used to denote a *fully developed* schizont in which the merozoites are differentiated.

Trophozoites.—When the sporozoites are introduced into the body of the human by the mosquito it is believed that a stage of development takes place which is not yet completely understood. The parasites do not appear in the circulating blood until several days after the bite of the mosquito. Those of aestivo-autumnal malaria have been

found as early as 6 days after inoculation, but those of benign tertian never earlier than the eighth day (4). Wenyon estimates that it usually takes time enough for five cycles of the parasite before definite symptoms of the disease appear. In benign tertian this is about 10 to 12 days; in aestivo-autumnal about 8 to 12 days, and in quartan about 21 days.

The parasite when it has entered [1] the red blood cell grows, using the substance of the cell as food. In about five to six hours after the cell is infected, fine yellow or brown granules of pigment begin to appear in the cytoplasm of the parasite. These pigment granules probably represent chiefly the byproducts of the digestion of the hemoglobin and are frequently called haemozoin. In smears stained with Giemsa's, Wright's, or Leishman's stains, the chromatin of malaria parasites stains dark or purplish red, the cytoplasm stains blue, and the pigment granules vary with the species from golden brown to almost black.

The youngest form of the parasite seen in the peripheral blood in all of the species is termed the "*signet ring*" from its appearance—a small dot of chromatin usually on the periphery of a circle of cytoplasm surrounding a central vacuole. In many cases, probably, the appearance is due to the fact that an optical section of a sphere is seen, the protoplasm enclosing the vacuole all around although it is scarcely apparent on the upper and lower surfaces owing to its tenuity. Sometimes the chromatin dot is seen in the center of the circle of cytoplasm. Here again, however, it is probable that in many of these cases, the chromatin mass is really in the cytoplasm of the upper or under surface of the sphere. There may be more than one chromatin granule in the ring stage and this division is thought to be fragmentation rather than a division of the chromatin prior to schizogony.

The *growth* of the parasite is a *gradual* one, with some slight differences in the species, both in the parasite and in the effect on the cell. Malaria parasites in the trophozoite stage perform ameboid movements and ingest food supposedly by osmosis. After the parasite has used up the available food in the red cell and has thus reached *maturity* so far as vegetative growth is concerned, it prepares for asexual reproduction by gradually slowing and finally ceasing its ameboid movement, thus assuming a more compact form, often with an irregular outline.

Schizonts.—After maturity is reached the chromatin of the trophozoite divides into two parts and there is *progressive segmentation* until from 6 to 24 chromatin masses are produced, the number depending on the species of the parasite. As this division takes place, the cytoplasm also breaks up into parts, one part finally accompanying each

[1] According to some authors, parasites in some stages may be attached to the outside of the red blood cells.

small chromatin mass. These divisions, composed of chromatin and cytoplasm, are termed *merozoites*.

There is a tendency on the part of the pigment in benign tertian and quartan malarias to begin to aggregate as the chromatin begins division and to continue collecting until this division is completed, when all the granules are clumped in one or two places. In aestivo-autumnal malaria the pigment clumps early—often before any division of the chromatin (this being a differential characteristic when these stages are found).

Shortly after division is completed, the schizont bursts the red blood corpuscle, the merozoites are liberated into the blood along with the pigment, a possible residue of cytoplasm, and possibly toxic materials produced by the parasite. It is at this time that the person affected is likely to have the paroxysm or chill, followed by fever, characteristic of malaria. The pigment and the residual cytoplasm are phagocytized in the peripheral blood by the leukocytes—usually the monocytes, though sometimes by the neutrophils also. It has been stated that the neutrophils of the Negro are phagocytic to this pigment more often than those of the white person. Not all the merozoites survive after the bursting of the cell, but those which do are found shortly thereafter in or on the red blood cells. Here the designation "trophozoites" is begun again and here they start again the asexual cycle. As the disease continues the number of parasites is increased by geometrical progression until frequently enormous numbers develop. Sometimes more than one parasite attacks and enters a single cell.

Gametocytes.—While some of the merozoites continue the asexual phase others develop into sexual forms called *gametocytes*. Two kinds are formed, the female or macrogametocyte, and the male or microgametocyte. After entering the red blood cell, the merozoite which is to develop into a gametocyte becomes rounded off into a small compact body which stains blue with a red granule of chromatin. The chromatin dot may be surrounded by a colorless halo which is presumably the unstained part of the nucleus; but the parasite does not develop a vacuole and so does not assume the signet ring form. For some reason not properly understood, the growth of the gametocytes of all the species takes place almost entirely in the vessels of the spleen or bone marrow, but some immature gametocytes are at times seen in the peripheral blood. Growth of the gametocyte is much slower than that of the schizont, being thought to require nearly twice as long to reach maturity. In benign tertian and quartan malaria the gametocyte grows steadily as a rounded or ovoid compact body; but in aestivo-autumnal malaria the young gametocyte often assumes a more or less angular shape and gradually takes on the characteristic sausage or crescent shape as it matures. The gametocyte remains within the

membrane of the red cell for the period of its life in the blood of man, which is thought to be only a few days; it degenerates and dies unless taken up by the mosquito host.

II. THE TYPES OF BLOOD FILMS AND ADVANTAGES OF EACH IN MALARIA DIAGNOSIS

The Thin Film

The thin film, long familiar to the student of malaria, is ideal for the study of the morphology of the individual parasites of the various species of malaria, when the infection is so heavy that parasites can be found without a long search. In addition it shows the accompanying blood picture and it is entirely adequate for diagnosis if the infection is a heavy one. A thorough working knowledge of the thin blood film, i. e., the appearance of the normal constituents of blood, of the more common pathological changes in the blood cells, as well as of the different species of *Plasmodia* in their various stages, is necessary before attempting to learn to identify malaria parasites in a thick film. The thin film has the great disadvantage, however, of failing to reveal a great number of positive cases—particularly of light infections.

The Thick Film

The thick film is a method by which a relatively large quantity of blood is placed in a small area and stained so that the hemoglobin is dissolved from the red cells and the blood smear is rendered sufficiently transparent for examination by transmitted light. Knowledge of the procedure is indispensable to the person who is to examine the blood for malaria (*5*). It reveals, comparatively quickly, sparse or scanty infections such as occur in new or chronic cases. There are occasions when immediate diagnosis is a matter of urgent necessity and frequently in these cases no certain information can be deduced from a blood examination by the thin film method, whereas the thick film taken at the same time will reveal parasites. Because of its efficiency in picking up cases with rare parasites, it gives a much more accurate idea of the incidence of malaria in a survey, and because great numbers of slides can be dehemoglobinized and stained at one time, it is particularly well adapted to surveys on given populations and to the use of State laboratories where large numbers of slides for malaria examination are received daily during the summer and fall. The thick film gives an idea of the degree of infection and can be a great help, because of the increased density of parasites, in identifying the type of malaria in cases where only one or two young forms can be found in the thin film. It gives a fairly good idea of the number of leukocytes and shows pigmented white cells more readily than the thin film. It also makes it possible to compare the number of para-

sites with the number of leukocytes and, together with the white cell count, permits a rough estimation of the parasite density.

Ross estimated that the thick film is 25 times better than the thin film for diagnosis and said that from 16 to 30 times as much blood could be examined in the same time (*6*). Since his time many calculations have been made as to the superiority of the thick film over the thin film, in the amount of blood per microscopic field; the increase in number or percentage of positives found in thick films, as compared to thin films; the amount of time saved in the examination of blood by the thick film method, and the length of time required to find the first parasite on thick and thin films. W. M. James said that there was at least 30 and often as much as 50 times as much blood available for examination in a thick film microscopic field as in a thin film field (*7*). Schüffner and Swellengrebel said that there is from 10 to 50 times as much [quoted by Sinton and Banerjea (*8*)]. H. C. Clark, in examination of 1,431 bloods, found 45 percent positive in the thick, as against 17.5 percent in the thin film (*9*). Von Ezdorf's assistants found in 3,613 smears more than 4 times as many positives in the thick as in the thin, and the time consumed in examining the thick films was only about one-sixth of that used on the thin (*10*). Sinton and Banerjea found 7.7 times as many benign tertian cases and 22 times as many aestivo-autumnal cases in thick films as in thin films, in 2-minute intervals of examination; and the time required to find the first parasite favored the thick film as 1:6.6 in benign tertian and as 1:23 in aestivo-autumnal. Muehlens in a number of slides found 131 cases positive in both thick and thin films and 102 positives in the thick, but negative in the thin (*8*). Dempwolff in indexes found about 3 times as many positives with the thick as with the thin (*8*). As for clinical cases, W. M. James diagnosed 94 percent in thick as against 58 percent in the thin in one specimen each, quoted by Johnson (*11*). F. B. Johnson in a study of 749 cases of malaria found 95.5 percent positive in thick films as against 62 percent in thin films by single slide examinations (*11*). Krauss out of 657 clinical cases found 91 percent positive in thick films (*12*).

Insofar as examination time is concerned it has been estimated that 30 minutes is the minimum that should be spent on a thin film before calling it negative. This is based on the belief that fever is produced by one parasite per 100,000 red cells and it takes 30 minutes to scan 100,000 cells (*7*). Since so much more blood can be scanned in a single field in the thick film it is evident that the time is cut drastically. To quote Barber (*13*),"The time spent varies with the examiner, however, for it is difficult to standardize skill, experience, and above all conscientiousness." In the Public Health Service laboratory we prefer to use as a criterion a certain number of microscopic fields covered. In surveys, with fairly well-trained tech-

niemans, we limit the examination to 100 fields; in clinical cases we advise a longer examination if anything suggestive of parasitism is seen. The trained technician can examine 100 fields on a good thick film in 3 to 5 minutes and experience has shown that the first parasite is most often found in the first 20 or 30 fields.

Practice and experience are necessary to become proficient in the interpreting of thick films for they are different in appearance from thin films and, at first, may be confusing. However, the ease with which parasites can be found in the thick film, after a long unfruitful search by the thin-film method, is ample recompense for the time spent in learning the technique. It has been repeatedly observed that when a person has learned this technique, he has no desire to return to the thin film for diagnosis.

In nearly all cases parasites will be found in thick films from patients who show active clinical symptoms of malaria. Parasites may be reduced however to a microscopically undetectable level by antimalarial drugs. Also, in persons with extreme susceptibility symptoms may occur before parasites can be found. In these latter cases examination on subsequent smears should be made on successive days.

The thick film can be used for examination of blood for trypanosomes, filaria, the spirochetes of relapsing fever, and for estimation of the percentage of eosinophils. On two occasions *Herpetomonas muscæ domesticæ*, contaminants from the feces of flies, have been observed in thick films taken during field surveys and examined in this laboratory. Also, there has been seen in quite a number of thick films from various parts of the country small microfilaria which are as yet unclassified. Only one of these has ever been found on any one thick film and attempts to find them in later thick films from the same persons have been unsuccessful, so one cannot say whether they were in the blood when it was taken or were contaminations.

III. IDENTIFICATION OF THE SPECIES OF MALARIA IN THE THIN FILM

Plasmodium vivax (Benign Tertian)

Considering the parasites as they appear in stained thin films from peripheral blood, the youngest ring form consisting of a blue margin of cytoplasm and a rather heavy red dot of chromatin, which may be located centrally or peripherally, is about one-third the diameter of a normal red blood cell. After a few hours the red blood cells, infected with tertian parasites, are always enlarged, pale, and may be very bizarre in shape. Also, in correctly stained films Schüffner's stippling[2]

[2] This consists of bright pink granules which appear evenly distributed throughout the part of the parasitized cell not occupied by the parasite. The dots are rather consistently fine and uniform in size. As the parasite grows the dots often become more pronounced, and take a somewhat deeper stain. This stippling is peculiar to benign tertian and ovale malarias, hence is of diagnostic value. Careful staining is necessary to demonstrate the dots in the maximum number of infected cells and prolonged washing will obliterate them. Their exact nature is unknown (*14*).

may be demonstrated in many of the parasitized cells at any stage after this period. As development proceeds the parasite may continue to show a ringlike appearance in stained films with much thickened cytoplasm and enlarged chromatin mass. However, it may very early exhibit pseudopodial processes indicative of ameboid movement, a characteristic which is very pronounced in benign tertian malaria and which gave rise to the parasite's specific name "vivax." After 5 or 6 hours the trophozoite begins to show yellowish-brown pigment granules. These are small, angular or rodlike, and increase in number with the growth of the parasite. In the young forms they frequently cannot be distinguished as separate granules or rods, but exhibit their presence by giving a yellowish tinge to portions of the cytoplasm. As the trophozoite develops, it may assume practically any shape within the enlarged cell, with projecting pseudopodia and one or several vacuoles. Meanwhile both cytoplasm and chromatin are increasing in amount. In vivax this stage is far larger than the corresponding stage of the other species. At the end of about forty hours the parasite fills, or practically fills the cell, which may be twice its original size. It has now completed its vegetative growth and prepares for reproduction. To this end it draws in its pseudopodia, ceases its movement, assumes a rather compact form, usually irregular in outline and with cytoplasm mottled in appearance as though unevenly massed. It still has a single nucleus which is compact and usually lies near the periphery of the parasite. This stage may appear much smaller than some of the ameboid forms which have preceded it, and due to its compactness it usually stains much more heavily.

Now the division of the chromatin begins. First, there are two divisions possibly by binary fission, and then successive divisions until there are 12 to 24 segments, with 16 as a common number. At first these are irregular in shape but as division becomes complete the segments appear more regular in size and shape and rather small and compact, as compared to earlier appearances. In the meantime the single mass of cytoplasm has gradually broken up, one portion adhering to each dot of chromatin, thus forming individual parasites. These are the merozoites. During this process the pigment has gradually collected into one or two loose masses, the complete but rather loose clumping of the pigment being a definite sign that segmentation in this species is complete. Frequently there is an uneven number of merozoites in the mature schizont. The entire growth from small trophozoite through mature schizont requires 48 hours. Upon maturity the cell bursts, the merozoites attack new cells and begin another generation.

The majority of parasites attain maturity at about the same time. Not all do, however; some are earlier in maturing than others. Hence

there are often several stages of the benign tertian parasite in the peripheral circulation at the same time.

Gametocytes of benign tertian are sometimes found with the appearance of the first parasites in the peripheral blood, but more frequently they appear after schizogony has continued through several generations (*15*). It is thought that development of the gametocyte requires much longer than that of the asexual form. It has also been suggested that the gametocytes of *Plasmodia* may go through an intermediary step involving the production of premacrogametocytes and premicrogametocytes, before the final gametocytes develop. This might account for the additional time required (*16*). The shape of the growing gametocyte changes very little from the compact merozoite form, due to lack of the ameboid activity found in the trophozoite. The mature microgametocyte is often about the size of a normal red cell; the mature macrogametocyte is distinctly larger, sometimes almost double the size of a normal red cell. Both are in enlarged red cells just as mature schizonts are. The quantity of pigment granules in the mature gametocytes of both sexes is usually greater than in the schizont, and the grains and rods are usually darker in color in the female. They are always distributed rather evenly over the cytoplasm in both sexes.

The macrogametocyte possesses a densely blue-staining, generally homogeneous cytoplasm. The nucleus is usually compact, and very rich in deep red chromatin. Around this chromatin there is sometimes a colorless area which is called the zone of carolymph and is thought to be the nonstaining part of the nucleus. The nucleus is usually situated near the periphery of the parasite.

The microgametocyte contains less cytoplasm than the macrogametocyte; it stains more lightly than that of the female and may be gray-blue, greenish blue, pinkish blue, or at times practically colorless. There is a loose nuclear system with reticular distribution of the chromatin. Sometimes the nucleus is termed "stellate"; sometimes it extends in a broad spindle across the body. Practically always it is centrally placed and has a light staining quality. There is often in the microgametocyte the unstained vesicular area around the mass or grains of stained chromatin.

There is difficulty in differentiating between the full-grown trophozoite (just before division of the chromatin) and the slightly immature macrogametocytes. These points can be of assistance: The cytoplasm of the trophozoite may present a mottled appearance or may even contain one or more vacuoles; the outline is usually irregular and may show deep indentations. The pigment, although scattered through the cytoplasm, is in small golden-brown grains. In the macrogametocyte the cytoplasm is rather homogeneous and contains no vacuoles. The outline as a rule is regularly circular or ovoid. Its

pigment may be more abundant and in larger darker brown granules. The full-grown macrogametocyte is usually larger than the grown trophozoite.

Plasmodium malariae (Quartan)

The young trophozoites of quartan malaria are ring forms and are about the size of, or slightly smaller than, those of benign tertian, though they sometimes seem to have a broader circle of cytoplasm than young tertian rings. Double chromatin dots are rare. The vacuole of the ring stage disappears very soon after the parasite begins its growth. The growing stages may assume band forms which stretch across the red blood cell; but the trophozoites seen in stained smears may also be compact forms, angular or even round or ovoid in outline. The chromatin may be a rounded mass, though more frequently it is streaked or semi-circular in formation—even in the rounded parasites. There is very little ameboid activity in the growing and older trophozoites, hence one seldom finds the irregular, tenuous, ameboid forms that are so characteristic of benign tertian. As the parasite becomes older it may grow into a wider band form or have a more rounded or slightly irregular shape. Frequently, there is a peripheral arrangement of pigment, often along the edge opposite the nucleus. The pigment of quartan trophozoites appears early in the growth, is usually a darker brown than that of the trophozoites of benign tertian and the individual granules are usually larger. Mature forms of the parasite almost fill or completely fill the normal sized red cell and may be rounded or band shape with rounded or elongate chromatin mass. The cells containing quartan parasites are never enlarged—frequently they even seem smaller than normal and sometimes darker in the early stages. The completion of the cycle requires 72 hours, hence the quartan parasite grows rather slowly. When schizogony is complete there are from 6 to 12 merozoites (usually 8) sometimes arranged peripherally around the centrally clumped pigment (rosette formation), but more often in an irregular cluster.

Quartan malaria seems to have fewer gametocytes than the other two species. They are frequently very difficult to find. When the young gametocytes are found in the peripheral blood, they are very hard to distinguish from growing trophozoites which, in their compact form, resemble them very closely. The quartan gametocytes are smaller than those of benign tertian but their development follows the same course. Like those of benign tertian they are spherical or oval in shape and they have the same differences in sex and in staining qualities. The pigment is abundant, dark-brown, and coarser than in benign tertian. Growing gametocytes of quartan are said not to take the band formation (*17*). Mature macrogametocytes are likely to be larger than the mature trophozoites and they may be

ovoid in shape. The pigment granules in them are more numerous and prominent than in the trophozoites.

On occasions there have been demonstrated in the cells containing quartan parasites certain pink-staining dots similar to Maurer's spots in aestivo-autumnal malaria. These have been named Ziemann's stippling. Investigators seem to agree that the stippling is usually pale and the dots slightly irregular in size, round, and less distinct than in tertian. Also, they are, as a rule, less developed than those found in aestivo-autumnal. They seem to be best brought out by intensive staining or stain solution the pH of which is about 7.5. Since they have little differential diagnostic significance, it is thought to be sufficient in diagnosing smears to stain slides at the neutral point and ignore these possible granules.

P. malariae in the United States as a whole is the least often found of the species, but in some vicinities the comparative proportion of this species is rather high.

Plasmodium falciparum (Aestivo-Autumnal)

The small trophozoites of aestivo-autumnal malaria are usually smaller than those of the other types, have quite a delicate thread-like line of cytoplasm and one or more rather small dots of chromatin. Double chromatin dots are much more frequently found in this species than in the others. The rings may vary in size but in their youngest stage are usually smaller and much finer than the equivalent stage of benign tertian, and when found in sufficient numbers this small size and delicacy are leading diagnostic characteristics. The young parasites may at times be quite irregular in form (round, rectangular, flame-shaped, or streaked). Flattened marginal forms and bridge forms are more common in aestivo-autumnal malaria than in any other type. Multiple infections of the single cells are also more common in this species, though certainly not confined to it.

As growth proceeds the parasites of aestivo-autumnal malaria usually retain a ring formation much longer than most benign tertian parasites. The older rings differ from the very young ones in the slightly increased size and in the increased amount of cytoplasm and chromatin; also in the fact that they contain traces of pigment, which give a yellowish tinge to the cytoplasm. These parasites, which correspond in age to the large ameboid forms of benign tertian, may be confused, in a diagnosis of smears containing few parasites, with the younger ring forms of tertian, since they are so nearly the same size as these. Because aestivo-autumnal parasites do remain as ring forms during much of their early growth, and because of the great number of parasites produced in an aestivo-autumnal infection, there are likely to be more rings of aestivo-autumnal in the peripheral circulation at one time than in either of the other species. Also,

unlike *P. vivax* or *P. malariae*, it is the tendeney of the parasites of *P. falciparum* to disappear from the peripheral blood in the ring stage and eomplete their growth and development in the capillaries of the internal organs. This accounts for the fact that, of the stages of the asexual parasite, usually only the ring forms are found in the periph-eral blood. The exaet stage at whieh the ring forms disappear from the circulation varies; sometimes they disappear very soon after they enter the red blood eells; at other times they remain for hours, attaining eonsiderable size. With the foregoing faets, *one may state that when only a large number of ring forms are seen and no older tro-phozoites or schizonts can be found, the infection is in all probability aestivo-autumnal.* Ring forms are found more readily after the ehill. In aestivo-autumnal malaria, if the ease is running a tertian course, the parasite count will be much higher during the period of inter-mission between paroxysms than on the day of the paroxysm.

The more mature trophozoites and schizonts are, as a rule, not found in the peripheral blood. However, in some heavy or intense infections rare ones may oeeur usually along with large numbers of ring forms. The old trophozoites, when they are found, eonsist of a small bit of compaet, light-staining eytoplasm, a granule of chromatin somewhat larger than in the ring and usually a dull blur or a small, dense, almost blaek block of pigment. The parasite at this stage is very small, sometimes hardly as large in eireumference as the older ring forms. The presegmenting and segmented sehizonts resemble those of the other species; but even in its complete segmentation the aestivo-autumnal parasite fills only about two-thirds the diameter of the normal red eell. To differentiate the older forms of aestivo-autumnal from those of quartan, which they most resemble, one may keep in mind the following faets: the respective stages of aestivo-autumnal are smaller, and they contain less pigment, whieh is usually black or almost black in appearance, and has a tendency toward early clumping (often before the division of the ehromatin begins).

There are from 8 to 24 divisions of the ehromatin in aestivo-autumnal and about 48 hours are required for maturation. The cell attaeked by an aestivo-autumnal parasite is not altered in size. Sometimes there may be observed in the eells eontaining the parasites red staining dots ealled "Maurer's spots." They are irregular in shape and size, though they are generally much coarser than the dots of Schüffner's stippling and not nearly so numerous. They are brought out in some slides with overstaining or with the staining solution on the alkaline side. Some films show them far better than others. The age of the parasite may have some effeet on their staining.

The gametocytes of aestivo-autumnal are quite different from those of the other two species exeept in their youngest stage; at that time they are small compact bodies of cytoplasm with a small mass of

chromatin, and like the other gametocytes they do not develop a vacuole. Their shape, from a rather early stage, however, is likely to be elongate or angular though rounded forms are found also. As the gametocyte grows the chromatin extends in a thick line, usually along one side of the body. The pigment in this stage is scattered through the cytoplasm. Occasionally, one finds long, thin spindle-shaped or lanceolate forms in which the pigment is rather evenly distributed throughout the length of the parasite. These are young gametocytes, also. As the gametocyte gets older the chromatin and pigment generally pass to the center of the body and the parasite assumes an elliptical, slightly crescentic or sausage shape which is different from anything found in the other species.

The mature macrogametocyte shows a dense blue cytoplasm and a compact, small red mass of chromatin, lying in or near the center, or near one of the poles. The pigment, in separate grains, closely adheres to the chromatin, surrounding it completely or on one side, sometimes even covering it. The macrogametocyte is often more slender and slightly longer, as well as more deeply stained than the microgametocyte. The microgametocyte, in comparison, is likely to be broader, shorter, and more sausage-shaped, with lighter staining qualities throughout. The cytoplasm in the microgametocyte is usually pale, often grayish-blue or pink, the chromatin is loose and scattered, with the abundant brownish pigment rodlets and granules, irregularly through about half of the length of the crescent. As a rule, only mature crescents are found in the peripheral blood, but immature ones like the older asexual forms may be found in the circulating blood of persons seriously affected.

It is usually days before gametocytes of aestivo-autumnal malaria appear in fresh infections. On the other hand, in old or chronic cases, they are frequently found when no rings are to be seen. The gametocytes of aestivo-autumnal malaria seem to be produced in showers at intervals during the disease instead of being produced in proportionate number simultaneously with the asexual forms as in benign tertian malaria.

The cells containing the crescent forms stretch as the longitudinal growth proceeds and frequently one can see in the stained thin film, on the concave side of the parasite, a faint bow-shaped projecting rim, representing the residuum of the infected red cell. Sometimes the remains of the red cell appear as a red zone around the crescent.

Plasmodium ovale

Most of the cases of malaria caused by *P. ovale* (*18*) have been reported from East Africa, though there are records of two cases in South America. So far none has been found in the United States. Those persons who have seen and worked with *P. ovale* advise micro-

scopical comparison of well stained thin films of the classical species with some of this species in its various stages, in order to ascertain those characteristics which distinguish it. They state definitely that it cannot be identified in the thick film because of resemblance to both vivax and malariae parasites. As in vivax, the parasite produces a tertian fever; i. e., the complete asexual cycle requires approximately 48 hours. As with vivax, the infected cell shows Schüffner's stippling and enlarges as the parasite grows older. As in both vivax and malariae all stages of the parasite appear in the peripheral blood.

Considering the young ring stage, the most definite means of identification seems to be a thick studding of Schüffner's stippling in almost 100 percent of the infected cells. According to James, Nicol and Shute (*18*) these dots in ovale are numerous, even with the very young stages and are large and well defined, as compared with the rather sparse, fine dots found in a few of the cells infected with young forms of vivax. The ring forms are, as a rule, darker in color and more solid than those of falciparum.

In the older trophozoite stages of ovale the parasite has a striking resemblance to that of *P. malariae*, since it has little ameboid activity. In actual measurement, it is larger and has less pigment, which is lighter in color and not so conspicuous as that of malariae. A large proportion of the infected cells containing this stage and succeeding stages will be found to be rather longer than wide and to have an irregular fringed, not well defined margin drawn out into ragged points. The deeply stained Schüffner's stippling fills the cell even to the ends of these transparent points. While such cells might be found upon long search in vivax, they are frequent enough in ovale to be characteristic of the species.

The presegmenting stage resembles closely the same stage of quartan, but it is a rounded parasite found in a definitely oval-shaped enlarged cell, which may have the fringed edge spoken of above and usually has heavy Schüffner's stippling.

The segmented schizont, as in malariae, usually has eight segments often arranged in rosette form; the infected cell is enlarged, may be oval and fringed, and it nearly always has the heavy Schüffner's stippling.

The sexual forms resemble those of vivax and are difficult to distinguish from them. They are distinguished from malariae by the enlarged cell and presence of Schüffner's dots.

Mixed Infections

It is believed that there are many more mixed infections than are usually determined in blood examinations, and that the possibility of a mixed infection is frequently overlooked by the microscopist. First of all, it seems to be the tendency in mixed infections for one species to predominate over the other (*19*), and the chance of finding numbers of the parasites of one species in a film and of missing completely the rare parasite of another species is quite possible. The fact is that many technicians look no further when definite parasites of a single species are seen. Of course, typical and characteristic forms of the respective species must be found to determine a mixed infection. In thin films these specifically distinctive forms in aestivo-autumnal malaria are the crescent-shaped gametocytes. In benign tertian they are the large, distinctly ameboid trophozoites, or any stage of the parasite in an enlarged cell containing Schüffner's stippling. In quartan malaria they are the pigmented band forms or any heavily pigmented form beyond the ring stage in an unenlarged cell. The mixed infections in the United States are most frequently aestivo-autumnal and benign tertian; sometimes however they are benign tertian and quartan, aestivo-autumnal and quartan, or even, on rare occasions, all three species.[3]

Accompanying Blood Picture

In malaria the total number of white cells, as a rule, is below normal, though the count may rise during the febrile period, or with some complicating factor such as pneumonia. In the stained thin film one frequently finds the picture of secondary anemia in greater or less degree; i. e., there may be increased polychromatophilia or basophilic stippling, central achromia of the red cells, variation in their size and shape, or even nucleated red cells. In the early stages of the disease there may be a reduction of the number of neutrophiles and an increase in lymphocytes and monocytes. During subsequent attacks there is a transient increase of neutrophiles with a display of many immature forms. After the attacks the neutrophiles decrease again and the monocytes increase. These latter play a part in combating the infection by ingesting the pigment and damaged cells. Clumps of malaria pigment in the white cells are almost as certain proof of malaria as the parasites themselves, but when this pigment is present, one usually finds plenty of malaria parasites, also.

[3] This information on parasites in the thin film is based partly on experience and partly on information obtained from Thompson and Woodcock (*77*) Wenyon (*35*) Stitt, Clough & Clough (*36*) Nocht and Mayer (*37*) and James, Nicol and Shute (*18*).

Summary of Parasite Differentiation

	Stained thin films			
	Plasmodium vivax	*Plasmodium malariae*	*Plasmodium falciparum*	*Plasmodium ovale*
Infected cell	Larger than normal, pale, often bizarre in shape. Schüffner's dots very often present. Multiple infection of erythrocyte not uncommon.	About normal or slightly smaller. Sometimes darker in early stages. Ziemann's dots rarely seen. Multiple infection of erythroeyte rare.	Normal in size. Multiple infection of erythrocytes more frequent than in the other species. Maurer's dots sometimes seen (in overstained smears or when pH of H_2O is on alkaline side).	Somewhat larger than normal, often with fringed or irregular edge and oval in shape. Schüffner's dots appear even with younger stages, stain more readily and more deeply than in vivax.
Small trophozoite (early rings).	Signet ring form with heavy chromatin dot and large cytoplasmic circle, possibly with fine pseudopodia.	Signet ring form with heavy chromatin dot and cytoplasmic circle which is often smaller, thicker, and heavier than that of vivax.	Small threadlike cytoplasmic circle, and one or two small chromatin dots. (Double chromatin dots more frequent than in other species). Marginal and bridge forms are frequent. May disappear in this stage from peripheral circulation and return to internal organs for development.	Small, darker in color and more solid, as a rule, than those of falciparum. Schüffner's dots regularly present in almost 100% of infected cells.
Growing trophozoite	Same as above with gradual increase in amount of cytoplasm and chromatin. Often with more distinct pseudopodial processes. Small yellowish-brown pigment granules in cytoplasm, number increasing with age of parasite.	Chromatin rounded or elongated, cytoplasm in a compact form with little or no vacuole or in a narrow band form across the cell. Dark brown pigment granules—may have peripheral arrangement.	This stage remains in the ring form but chromatin and cytoplasm increase to the extent that in size the parasite resembles closely the small trophozoite of vivax. A few pigment granules give a yellowish tinge to the cytoplasm. This is usually the oldest asexual stage seen in peripheral circulation.	Resembles closely same stage of *P. malariae*, but is considerably larger. Pigment is lighter in color and less conspicuous.
Large trophozoite	One abundant mass of chromatin, loose, irregular or close compact cytoplasm, with increasing amounts of fine brown pigment. Parasite practically fills enlarged cell by end of 36 to 40 hours.	One mass of chromatin, often elongated, frequently less definite in outline than that of vivax. Cytoplasm dense, compact with few irregularities of outline; in rounded, oblong or sometimes band shape. Pigment granules larger, darker than in vivax with great tendency toward peripheral arrangement. Fills or almost fills normal cell.	Stage seldom seen in peripheral blood. Very small, solid, with one small mass of chromatin; lightly staining, compact cytoplasm; and with a haze of pigment scattered through the cytoplasm or with very dark pigment collected in one small, dense block.	
Schizont (presegmenting)	Chromatin divided into a number of masses; cytoplasm shows varying degrees of separation into strands and particles; pigment shows tendency to collect in parts of the parasite.	Same as vivax except that the parasite is smaller and shows fewer divisions of chromatin, as it approaches segmentation, and more delayed clumping of pigment.	When found in peripheral blood this stage resembles the same stage of *P. malariae* but is smaller and the pigment is likely to be completely clumped in one small dark mass.	About 25% of infected cells are definitely of oval shape. Usual picture is that of a round parasite in center of an oval cell. Many cells with indefinite fringed outline. Pigment lighter in color and less coarse than in *P. malariae*.

Mature (schizont)	12 to 24 divisions or merozoites, composed of a dot of chromatin and a portion of cytoplasm. The pigment is in one or two clumps. Parasite practically fills enlarged cell.	6 to 12, usually 8 or 10 merozoites in a rosette or irregular cluster. Practically fills normal-sized cell.	8 to 24 merozoites, which are very small compared to those of other species. Rarely found in peripheral blood. Fills about two-thirds of normal-sized cell.	Usually 8 merozoites arranged around a central block of pigment.
Macrogametocyte	Dark blue, homogeneous cytoplasm with no vacuoles; small, compact, dark red, usually eccentric chromatin; abundant dark brown pigment scattered through cytoplasm. When grown usually fills or nearly fills enlarged cell. The outline is circular or ovoid and regular.	Cytoplasm and chromatin same as vivax. Pigment is abundant, dark brown, coarser than in vivax. When grown usually fills the normal sized cell. Outline circular or ovoid.	Cytoplasm possibly a deeper blue than in microgametocyte. Usually single dark red chromatin mass near center associated with concentrated aggregation of pigment, darker than in microgametocyte. Crescentic or sausage-shaped about 1½ times diameter of erythrocyte in length, possibly longer and more slender than microgametocyte.	Distinguished from *P. malariae* by size of infected cells and by Schüffner's dots. Less easy to differentiate from vivax. Seldom or never contained in an oval erythrocyte.
Microgametocyte	Light blue, gray, pink or almost colorless cytoplasm; large diffuse light red or pink chromatin—usually centrally placed, often with vesicular area around chromatin mass. Abundant yellowish-brown pigment throughout cytoplasm. When grown, about size of a normal cell. Usually circular in outline.	Same as vivax except in size. When grown, fills or almost fills normal sized cell.	Often the cytoplasm is paler than in macrogametocyte—grayish blue or pink. Loose, diffuse, light staining granules or threads of chromatin scattered with numerous granules of pigment throughout central half or more of parasite. Parasite possibly broader, shorter, and with more rounded ends than those of macrogametocyte.	
Length of asexual cycle	48 hours	72 hours	48 hours	48 hours.
Stages in peripheral blood	All	All	Usually ring form trophozoites and gametocytes. Other stages rarely found except in severe cases.	All.
Remarks	More stages of growth likely to be seen in one film than in other species. Gametocytes appear early in cycle.	Parasites are usually more compact and hence appear more intensely stained than those of other common species. Gametocytes rarer than in other species, appear late. Least often found of 3 species in United States.	Parasites frequently more numerous than in other infections. Unlike other species—growth of asexual forms, following the ring stage, take place in internal organs.	Species not found to date in United States. Differentiation not possible in thick films.

IV. MALARIA PARASITES IN THE THICK FILM

Technical Procedures

MAKING THE FILM:

1. *For making thick films use only slides which are clear, unscratched, noncorroded, and above all meticulously clean—i. e., free from grease, dust, acid, or alkali.*

When slides are to be cleaned and reused repeatedly a grade of glass should be used which will stand the cleaning process and the necessary handling without fogging or corroding, and which will not scratch too easily. Scratches in slides will hold the stain and give various false impressions; corroded slides have this disadvantage as well as that of cutting out part of the light necessary for exact microscopical work. New slides should never be used without cleaning, no matter how bright or shiny they may look, for there may be left on them traces of oil put there in the glass polishing process and this will cause difficulty. Thin films will not spread evenly on an oily or dirty surface and thick films will not adhere to it. There may be on an unwashed new slide traces of soda, lime, or potash from the glass itself, and these particles can change the pH of the stain to such an extent as to make young malaria parasites and parts of older ones invisible. Great care must be exercised in handling clean slides, since oil from the fingers on the slide's surface will cause thick smears to slip off in staining.

For new slides the following process may be used: Wash the slides with warm water and mild soap, rinse well in warm running tap water, then in distilled water, dip in ethyl alcohol, (preferably 90 or 95 percent) and dry with a clean, lintless cloth. They will not be scratched if handled with reasonable care. After slides are cleaned they should be protected from dust. To accomplish this, groups of 25 slides or more may be wrapped in roll toilet tissue and bound with a rubber band. These packs are particularly convenient for field survey work, or they may be used in the laboratory. For the practicing physician, who carries slides in his bag, it is suggested that he wrap them slide over slide so that the surface of only one slide at a time is exposed in unwrapping. This will keep the remaining slides clean for future use.

For cleaning used slides on which mineral oil has been used for immersion, the following method has been found quite satisfactory: Immerse the slides for about 12 hours in dichromate cleaning solution. Rinse them well in running tap water. Let them stand in a solution of warm soapy water for about an hour. Rinse the slides again in warm running tap water. Rinse in distilled water. Let stand in alcohol for a short period. Dry and shine with a soft lintless cloth

2. *Be sure that the blood taken is free from grease, perspiration, or dirt which may be on the skin, and that the alcohol used for cleansing never be allowed to mix with the blood.*

Cleanse the area to be punctured with gauze or cotton soaked in

alcohol. Then rub dry with a piece of sterilized cotton or gauze (gauze is to be preferred since it does not leave lint on the skin). Alcohol on the skin or needle will "fix" the red blood cells and interfere with the dehemoglobinization of the red cells which is part of the staining process. Hence all alcohol must be wiped away or allowed to evaporate before blood is taken for the smear. Some workers prefer to prick through the alcohol on the skin and then wipe away the alcohol with the first drop of blood.

3. *Prick the skin deeply enough to allow the blood to well up in a large drop under gentle pressure but not deeply enough to cause excessive bleeding.*

Every one has a favorite instrument for this purpose. A needle with a pyramidal point has these advantages: it cuts as it pricks and the puncture consequently can be very shallow and practically painless, yet give sufficient blood for a good thick film without squeezing.

4. *Near one end of the slide cover a space about the size of a dime with as much blood as will easily spread over this area without crackling and peeling when dry.*

This smear of about 3 to 5 average drops may be made in either of two ways: First method is to touch the under surface of the slide to the crest of the large rotund drop of blood and without losing contact with the drop of blood or touching the finger move the slide in narrow circles in the blood until a smear of the required size and thickness is made. The second method is to take several average size drops of blood quite near each other on the slide, and then, with the needle or with the corner of a clean slide, quickly puddle these into one fairly homogeneous drop about the size of a dime. One should be careful to take enough blood to make a smear which is several layers of erythrocytes thick and yet not so thick that it will contract and pull loose from the slide in drying. On the other hand, one should be sure not to make the smear too thin, else it will have no advantage over the thin film. Never use the second method without smearing the drops together and do not put them so far apart that the blood has to be spread over a large thin area in order to bring them together. The ideal thick film is several layers of erythrocytes thick in the middle and has a thinner edge of one-cell thickness. Ordinary printing can just be read through the wet center of a well-made thick film when the slide is placed over the printed page. The smear is placed near one end of the slide (its edge about ¼ inch from the end of the slide) so that only a small amount of stain will be needed to cover the smear with the slide vertically immersed in the solution.

5. *On the end of the slide opposite the blood film put an identifying number or character with a wax pencil or other marking device.*

This should correspond with the name or number on a data slip,

statistical record, or information blank, so that there will be no confusion in identifying the slide. Never write the number in the thick blood film, for this may cause the thickened blood to be loosened in places from the slide, or it may interfere with correct examination of the small area covered by the blood, sometimes actually obliterating the rare parasites. If a thin film is made on the same slide with the thick film, as is sometimes desirable, the number may be written on the thin film with a lead pencil.

6. *Lay the slide flat to dry so that the blood may be evenly distributed, and have it well protected from dust and insects. Air dry without application of excessive heat.*

If the blood smear is tilted while drying, the greater part of the blood will collect in a much thickened line along one edge of the smear. This may peel off completely in staining and, if not, it will be too thick for ease in examination. Also, the remainder of the film will be so thin as to be valueless, in light infections, for diagnosis. A covered Petri dish may be used for drying slides in the laboratory. In field work the slides should be inserted film-side down in a slide box (capacity of 25) which is held upright against the perpendicular of an inverted T block by means of a rubber band. (See plate XIII, No. 5.) When this box is full it should be closed and the box kept in an upright position until the slides are dry. Stained dust particles, etc., may cause trouble for the microscopist and slow down examination time. Flies or cockroaches will eat away the blood or contaminate it with bacteria or other organisms. If possible, let the blood smear dry in the air for 8 to 12 hours, thus protected. Thick films stain most clearly when several hours old. Very fresh smears may not have had time to adhere well to the slide and hence part of the blood may be lost. They frequently show a meshlike, fine fibrinous arrangement in the background also. This does not interfere materially with diagnosis but does not allow so clear a picture as the same slide will present if it dries for a few hours before staining. However, if a report must be made immediately the slide may be stained as soon as visibly dry, provided it is gently handled in both the stain and rinsing water. To aid in quick drying the slide may be placed for a short while in an incubator at 37° C. Too long drying of this kind, it is believed, will harden the cells and prevent perfect staining. Drying by means of warm air from an electric hand hair dryer held not too close to the wet smears, is an ingenious method that has been suggested and used satisfactorily (*20*). The stirring up of dust, etc., should be avoided if this method is employed. Direct excessive heat should never be applied for, like alcohol, it "fixes" the red blood cells.

7. *When smears are taken outside the laboratory they should be sent immediately to the person who is to stain them—because of the adverse effect of summer heat or of age on the smears.*

Slides shipped by mail should be sent either in the slide boxes (these in turn carefully packed in larger firmly made boxes), or should be wrapped *when dry*, slide over slide in toilet tissue in small packages. These are then placed with much protective packing in mailing containers to prevent breakage in shipping. The custom of dropping a slide into an envelope and mailing it, or sending slides in other ways in which they are easily broken in the mails, is deplorable. Broken slides are most difficult to stain and to examine, and no technician can do creditable work with such material.

Thick films may be hardened or partially fixed by age or by summer heat to the extent that they will not give up their hemoglobin. Such films are valueless. Old films never take the stain so easily or so brilliantly as fresh ones, but if they are not fixed by age, heat, or alcohol, they may be diagnosed provided they are carefully stained. Unstained slides may be successfully kept for a number of days during cool or cold weather and in our laboratory during the summer have been stored in a cold room over a week end. In the latter instance care should always be taken to prevent condensation of moisture on the slides, since this may loosen the smears and cause them to be lost during the staining process.

STAINING THE FILM:

1. *Stains.*—The most dependable stain, particularly for thick films, is obtained with a good quality of Giemsa stain solution diluted with distilled water of a pH from 7.0 to 7.2. Grübler's dyes have long given complete satisfaction in this work (*21*). When Giemsa stains made from American dyes are used, they should be those stains which are certified by the Commission for the Standardization of Biological Stains. Recently certified samples of American Giemsa will be more likely to conform to the needs of the malariologist, as the staining of malaria parasites is now one of the tests used by the Commission prior to certification of Giemsa stains. If the Giemsa stain solution is made from powders, the best reagent methyl alcohol (neutral, acetone free) and glycerine C. P. (neutral) should be used. The glassware used should be chemically clean and dry. For thin films some Wright's and Leishman's stains made from standard formulae will also give satisfactory results.

2. *Buffer solutions.*—Buffered distilled water for staining and washing ensures that the blue and red elements in the stain shall be taken up in correct degree by the blood and the parasites, thus giving maximum color contrast and facilitating diagnosis. To obtain a pH of from 7.0 to 7.2 in the water used for the dilution of the stock stain as well as for washing the stained slides, buffer solutions should be added to the distilled water. Disodium phosphate and either sodium or potassium acid phosphate in M/15 solutions are used and are prepared by dissolving the salts as follows: Na_2HPO_4 (anhydrous),

$M/15 = 9.5$ grams per liter; $NaH_2PO_4.H_2O$, $M/15 = 9.2$ grams per liter, and KH_2PO_4 $M/15 = 9.07$ grams per liter. The two stock solutions are kept in separate glass-stoppered pyrex bottles from which are removed the following quantities to make the indicated amount of the buffered water:

pH	$\frac{M}{15}Na_2HPO_4$	$\frac{M}{15}NaH_2PO_4.H_2O$ or $\frac{M}{15}KH_2PO_4$	Distilled H_2O
7.0	61.1 cc	38.9 cc	900 cc.
7.2	72.0 cc	28.0 cc	900 cc.

The pH of the buffered water is then tested and adjustments made if necessary. Brom-thymol blue indicator solution and a set of brom-thymol blue color standards are used for testing. Buffered water should be prepared each week. If this is inconvenient, the neutral water may be put into a large container provided with a pinch cock and tube, and the surface of this water may be covered with mineral oil to keep out the CO_2 from the atmosphere, which will charge the pH. Of course, the water should be replenished before the oily surface reaches the bottom of the glass tube.

3. *Making slides into blocks or groups for staining.*—If a number of slides are to be stained, the technician's time may be saved and uniformity in quality of staining assured by the simple method described by Barber and Komp (*22*). Slides are made into blocks of as many as 25 by placing inch squares of cardboard (about the thickness of the glass slide) between the slides at the ends opposite the blood films, compressing the alternating slides and squares and winding around that end of the block an inch wide strip of heavy paper which is secured with a strong rubber band. The paper prevents the slides from cutting the rubber band and the tight rubber band keeps the free ends of the slides from touching and assures access of the stain to the surface of all the thick films. The outer slides in the block should always be placed with the film side "in," to prevent the wet smears from being scraped off in the staining process. By this method hundreds of slides may be stained together, and consumption of stain diminished by using a container of the exact size necessary to hold the number of blocks to be stained. In survey work each pack may be identified by a cardboard square, containing necessary data, slipped under the rubber band on the outside of the pack. Even for a few slides this block method is quite convenient and economical.

4. *Longer staining process* (*particularly suited to surveys*).—Make up the amount of stain necessary for the staining container to be used, in the proportion of 50 parts of neutral distilled water to 1 part

of Giemsa stain solution. Set the slides on end in this stain and leave for 45 minutes. (An interval time clock is useful here, as it leaves the technician free for other tasks and prevents any chance of overstaining). At the end of this time remove the slides and set them in neutral distilled water for 3 to 5 minutes. (The age and thickness of the smear and density of the stain are the controlling factors here. Very fresh smears require little washing, as do films that are not very thick. Older, thicker, or deeply stained films give a clearer picture if washed a longer period of time.) Remove the slides from the water and allow to air dry standing on end on absorbent paper. Never blot thick films. An electric fan will facilitate the drying but direct heat is not advised, since some of the excess stain, which might flow from the smear, may be dried upon it and cloud the background.

To avoid expense use a staining dish holding the least amount of diluted stain which will cover the thick films when the slides are stood on end in the dish. Even the ordinary Coplin jar requires much more stain than is necessary for the number of slides which it will hold. For staining two to six slides, with cardboards between, an excellent staining dish can be made by filing off the top of an ounce straight-sided medicine bottle. In this container only 15 cc. of water and 0.3 cc. Giemsa stain solution are necessary.

Always clean the staining dish after use, as sediment from previous staining may precipitate part of subsequent stain. For maximum staining qualities use the stain only once.

Thin films may be stained in the same way as thick films and the stain is much more permanent than that of Wright or Leishman, but they must be fixed before staining with absolute methyl alcohol and *they cannot be washed after staining by standing in distilled water.* Washing is done by dipping the slides once or twice, for much of the detail obtained will be lost with the prolonged washing. Such slides are excellent for class study. They should be protected from the light when not in use.

If thick and thin films are put on the same slide, care must be exercised to keep the alcohol from touching the thick film and a compromise must be made in washing. It is probably better to have a little deeper background than usual in the thick film and retain the details of the thin, so dipping two or three times for washing is advised. A small cotton swab dipped in alcohol has been found satisfactory for fixing the thin films.

5. *Rapid staining method.*—To make the thick film technique meet the needs of physicians in their offices and diagnosticians in clinics and hospitals, we suggest the following method which permits rapid reporting on specimens and yet loses none of the advantages of the longer method when used on smears that are no more than a few hours old (*23*).

1. Dry the thick blood films in an incubator at 37° C. or with an electric hand hair dryer.

2. Make solution of Wright-Giemsa stain (*23*) (as prepared below) in proportion of 1 part of stain and 9 parts neutral distilled H_2O. Pour this over slides in a staining dish.

3. Stain slides for 10 minutes.

4. Flush scum from top of the dish with neutral water, then remove slides and wash them for 1 minute in neutral distilled H_2O.

5. Dry and examine with oil immersion.

PREPARATION OF WRIGHT-GIEMSA STAIN

To make Wright-Giemsa stain, dissolve 2 gm. Giemsa powder (National Aniline Chemical Co., Inc., New York) in 100 cc. glycerine (C. P. from a freshly opened bottle). This may be done by heating in a water bath at 55° to 60° C. for 2 hours and mixing well at intervals with a glass stirring rod.[4] To this mixture add 100 cc. Wright's stain solution (aged solution of 2 grams powder to 1,000 cc. methyl alcohol). Let stand over night and then add an additional 800 cc. of aged Wright's stain solution. Filter and use.

EXAMINING THE FILM:

1. *Equipment.*—A good microscope, preferably a binocular for constant work, equipped with 5 X or 6 X oculars for searching and 10 X for closer study of questionable objects, together with an oil immersion objective of 1.8 to 2 mm., and a microscope lamp giving sufficient blue white light are essential. The low power ocular gives a sense of greater distance, consequently is more restful to the eye. Also, it gives a larger microscopic field and so allows examination of more blood in a given number of fields.

2. *Immersion oil.*—For microscopic examination a heavy mineral oil or liquid petrolatum is recommended instead of cedar oil if positive smears are to be kept for reexamination. Mineral oil, after examination of the smear, can be carefully wiped away with softened lens paper without scratching the smear and the thin film of oil left on seems to protect the smear and preserve the colors for future examination. Of course, slides on which mineral oil is used must be kept free from dust if they are to be saved for demonstration later. Mineral oil has the disadvantage of not having the same refractive index as glass. The addition of alpha-brom-naphthaline will correct this, however. In tests made at the University of Michigan it was found that 18 parts of alpha-brom-naphthaline to 82 parts of "Nujol" seemed to be the proportions necessary to bring the refractive index very close to 1.51. To test the mixture, insert a glass rod into it.

[4] Avoid absorption of moisture by covering the mouth of the flask containing the mixture with a double thickness of paper secured with a rubber band and by removing the flask from the water each time the mixture is stirred.

If the rod is invisible the mixture is of exactly the same refractive index; if not, the proportions may be varied accordingly until the correction is made. A commercial product, Crown Immersion Oil, sold by Techni-Products Co. of Buffalo, New York, has the general appearance and all the advantages of mineral oil, in addition to which it has also the correct refractive index.

Xylol, for the removal of cedar oil, also removes part of the color from the smear, particularly from the parasites; and the use of cedar oil and xylol will give a "ground glass" appearance to the thick film through which it is impossible to distinguish parasites. Only one method has been found in this laboratory to remove this mottled, refractile surface when one wishes to reexamine such a slide. Place a generous amount of mineral or Crown oil evenly over the smear and warm it for 10 minutes or so over a very warm lamp bulb (such as is found in the better microscope lamps). The heat seems to cause a combination of the oils on the slide which clears the smear again for use. This oil is wiped away and a fresh drop of mineral oil placed on for examination.

3. *Outline for learning to examine thick films.*—Only well trained, conscientious microscopists can satisfactorily examine slides for malaria, and they have to reach perfection by degrees. This outline of study should be an aid:

(1) Learn the stages and species of malaria parasites in the thin film.

(2) Become familiar with the elements of nonparasitized blood in the stained thick film.

(3) Study *heavy infections* of various stages of known species of malaria in the thick film, comparing the stages of the parasite with those found in thin films taken on the same person at the same time. This study should always be begun in the thin edge of the thick film, where resemblance to the thin film is marked, and continued into the thicker portion with a comparison of the parasites found in the different areas.

(4) Study thick films containing *few* parasites and finally *rare* parasites and practice diagnosing on these slides, particularly for species. It is here that the real test of one's ability is made.

(5) For a while have a better trained thick film microscopist check all or some of your work for accuracy (a mechanical ringer on your microscope will be a great aid in marking objects on which you base your diagnosis, so that your checker may see the same objects which you see). An occasional check later will be an aid in maintaining a high standard.

(6) If you work with another microscopist, have the second person glance at all positives found while the identifying parasite is located. This promotes accuracy by admitting and encouraging discussion of uncertain objects which are encountered and gives both technicians the advantage of seeing all positives found.

Reporting

In reporting the results of examination of blood films for malaria one should not only specify, if possible, the species of parasites found, but should give some indication of the stages of the parasite and the

number found. Thus one may report estivo-autumnal, benign tertian, or quartan malaria, showing (many, few, or rare):

1. Trophozoites	a small b large
2. Schizonts	a presegmenting b segmented
3. Gametocytes	a microgametocytes b macrogametocytes

In the thick film we use the word "many" to designate the number of parasites when one finds them in every microscopic field; "few" when one finds them in every third to tenth field; and "rare" when one must search for them. A positive report can be made on one unmistakable parasite, but it is wise in cases of rare parasites to search carefully for additional forms to confirm the report. Often also, one picks up mixed infections by prolonging the search. The experienced technician can examine 100 fields on a good thick film in 3 to 5 minutes, and it has been shown that the first parasite is most often found within the first 20 to 30 fields.

Preservation of Specimens

Slides stained by the thick film method and examined with mineral oil or Crown oil, if protected from the light, will keep their colors for years. If one wishes to use these slides repeatedly, as for teaching purposes, they may be covered with glass slips in the following manner:

Place a large drop of heavy mineral oil or Crown immersion oil in the middle of the smear. Lower a very clean cover slip onto this drop. Then spread the oil to the edge of the slip and work out all bubbles by pressing on the cover slip from the center toward the edge with the eraser end of a pencil, or some other clean, resilient object. When the oil is spread evenly, clean all the excess from around the cover slip with a small artist's brush dipped in xylol and shaken. Do not let any xylol run under the slip. Then with another small brush, dipped in colorless lacquer, seal the cover slip on the slide by letting the lacquer dipped brush stroke overlap both slide and edge of cover slip. Allow the first coat to harden, then apply another, in order to make sure that no air can leak in to dry out the oil. If xylol touches the smear it will be partially decolorized; also if the mineral oil contains any acid, the same thing will happen. Slides thus covered may be cleaned and shined repeatedly with a piece of cleansing tissue or soft clean gauze, care being taken not to loosen or scratch away the lacquer.[5]

[5] Dr. Wendell Gingrich of University of Texas described before the American Society of Parasitologists on December 30, 1940, a simple method of preserving stained preparations using "Diaphane." His method of staining is different but it is believed that his method of preserving might be used on slides stained with the rapid Wright-Giemsa solution, or on slides stained with Giemsa alone.

Slides may be covered with paraffined paper also and the paper pulled off for examination.

General Appearance of the Blood in Stained Thick Films

The discussion of the differentiating points of the species of malaria is usually based upon their appearance in the thin film. In this kind of film the cells are spread over the slide in one layer and then by the application of alcohol, either as a separate process or in the stain itself, are fixed so that their outlines and the outlines of the parasites within them are preserved.

In the thick film, on the other hand, the blood cells are piled upon each other and removal of the hemoglobin from the cells is necessary to render the film sufficiently penetrable to transmitted light for microscopical examination. Dehemoglobinization takes place in the process of staining the unfixed film with an aqueous solution of Giemsa stain. The outlines of the red cells are thus obliterated and a microscopical picture presented which differs considerably from that of the thin film.

In the thicker portion of the thick film the background varies in color from a clear, light blue to a mottled gray-blue, depending upon stain factors, age of the smear, and individual blood variations; while at the edge, the thinner portion of the smear (many times only one cell in depth and about the width of one or two microscopical fields) often takes a pinkish color. Against the laked background the familiar purple nuclei of the white cells, in varying states of preservation, stand out clearly. Sometimes the cytoplasm of the white cells stains also, but it is ragged and uneven in appearance. The neutrophilic granules are often indistinct or absent but eosinophilic granules show rather distinctly in their characteristic color. Blood platelets are pinkish-violet in color, finely granular in texture and hazy in outline. They lie singly or in groups, and due to their distinctive granular appearance are not likely to be confused with parasites in the thick film. If the blood is taken from a free-flowing puncture there will be little likelihood of excessive clumping of platelets. Very often, particularly in anemic bloods, the thick film shows in the background (the thicker the smear, the more evident it is) nuclear and reticular remains of immature red cells (cellular debris). These may lie singly, often filling a space the exact size and shape of the laked cell, or they may appear in blue clouds of fine skein-like masses or stippling. See "Sources of Confusion or Error" p. 32. Barber claims that the degree of anemia can be estimated by the degree or density of these blue clouds. The technician with a well-grounded knowledge of the stages and species of malaria parasites, as they appear in the thin film, will have no great difficulty in diagnosing malaria in the thick film, or in differentiating species. For the less experienced person, the thin edge of the thick film will be very valuable for study. Here the red cells frequently retain a ghost-like outline and one finds characteristics of infected cells and of parasites duplicating those in the thin film—for

instance, the enlarged infected cells and distinct Schüffner's dots of benign tertian, band forms of quartan, marginal ring forms, doubly infected cells and perfectly formed gametocytes of aestivo-autumnal, etc. The thin edge is particularly valuable for determining mixed infections. It is well in learning thick films to start in this thin edge and work in toward the thicker portion comparing the typical forms with the less characteristic ones. Schüffner's stippling, with good staining of the smear and a short washing period, may frequently be seen as individual granules in the infected cells at the edge of the film, whereas, in the thicker portion, one sees sometimes a delicate pink area around the parasite which one interprets as the color caused by Schüffner's stippling. Maurer's and Ziemann's spots have not been observed in the thick film.

Except in the very thin edge the parasites usually appear without the definite outline of their host cells. The chromatin, cytoplasm and pigment stain characteristically, but in the thicker portion of the smear the parasites often seem smaller and more shrunken. This may be due in part to the destructive effects of lysis and also to the heaping and crowding of the red cells in the thick smear, which prevents the cells and parasites from flattening and spreading out as they do in the thin film. Also, in the slower drying thick film, the parasites have an opportunity to draw in their pseudopodia, which gives them in the dry film a denser, smaller appearance.

Almost constant focusing is necessary to distinguish all the parasites in the thicker fields, as the depth of the blood gives varying ocular planes. Too much stress cannot be laid upon careful staining, for ease in examination and accuracy of diagnosis are completely dependent upon it.

Appearance of Parasites in the Thick Film

Small trophozoites (rings).

In young ring forms the red or purplish-red of the chromatin dot first strikes the eye and the cytoplasm is distinguished subsequently. Young trophozoites may be found in the thick film in the signet ring formation just as in thin films, but are frequently not complete in outline; i. e., the chromatin dot with only a portion of the cytoplasmic circle is visible. This portion may be connected to the chromatin dot on both sides in short, straight or curved dashes, giving the impression that the rings are turned sideways or are lying at an angle to the plane of the blood. The very descriptive term "swallow form" has been applied to these (*24*). Often the cytoplasmic dash is visible adjoining only one side of the chromatin dot. This form resembles more or less an "exclamation mark" or "comma" and has been designated by these terms. Sometimes the cytoplasm follows a circular outline but is made up of seemingly disconnected small pieces

or fragments and is called an "interrupted ring." At times the cytoplasm is in a solid semicircular piece and lies completely disconnected from the chromatin dot on the curve of the ring opposite the nucleus. This form too might come under the term "interrupted ring."

When only this ring stage of the parasite is present and the rings are infrequent or rare in the thick film specimen, it is often impossible to differentiate the species. If an older form of the parasite can be found, this may facilitate the diagnosis.

In aestivo-autumnal malaria, the young ring forms are very small and delicate, the chromatin dot tiny, and the cytoplasm threadlike. If many small rings are found and no older form of the parasites is in evidence, one can be practically sure that the infection is aestivo-autumnal.

The young rings of benign tertian malaria are, as a rule, larger than the same stage of aestivo-autumnal with a heavier chromatin dot and cytoplasmic circle. Sometimes, even in this stage, there is evidence on the stained smear of ameboid activity shown by tiny pseudopodial processes on the cytoplasmic circle. It is seldom that species diagnosis must needs be based on young trophozoites alone, however, since older forms of benign tertian can usually be found (*25*).

The young ring of quartan malaria is often heavier than that of aestivo-autumnal and frequently not so large as that of benign tertian. In thick films it often shows as a large chromatin dot with a small concentrated mass or dash of cytoplasm. There is not so great a likelihood of the quartan ring spreading out or showing ameboid tendencies as there is in that of benign tertian. Barber has made the statement that he has never seen a smear of quartan malaria in which ring forms alone were present.

Growing and large trophozoites

While the young and delicate ring form of aestivo-autumnal, when found in sufficient numbers in the thick film, can often be differentiated from those of benign tertian and quartan, the growing trophozoites of aestivo-autumnal present more difficulty in diagnosis of species. These heavy ring forms of aestivo-autumnal are about the size of much younger forms of benign tertian and can be confused with the latter, yet they correspond in age to much larger ameboid parasites of *P. vivax*. The old trophozoite of aestivo-autumnal (the form just before the division of the chromatin), is not often found except in heavy infections and is consequently usually accompanied by large numbers of ring forms. When it is found, it is usually very small, compact, often nonvacuolated. The chromatin dot is larger than that in the young ring, the cytoplasm seems lighter than that of the similar stage of quartan, and the pigment, even at this stage, is usually clumped in one or two very small dark or blurred masses.

In thick films showing the growing trophozoites of benign tertian one may still encounter ring formations, much enlarged, with increased amounts of delicate, tenuous cytoplasm. There is a great variation in cytoplasmic pattern at this stage and there is a decided tendency in the older ameboid forms of this species for the cytoplasm to be fragmented and arranged irregularly in a cluster of varying-sized pieces with no visible connection. This cytoplasm is associated with a large round, or irregularly shaped, red or magenta mass of chromatin. The pigment appears as a yellowish haze, or as small light-brown grains or delicate rods on the cytoplasm. The trophozoite stage of benign tertian, just before division of the chromatin, is frequently quite solid in the thick film with a dark staining appearance often with a more or less regular outline. Its yellow-brown pigment, like that of the gametocyte, is rather evenly distributed through the cytoplasm. In the thick film these forms cannot be distinguished definitely from macrogametocytes.

Due to the fact that there is little ameboid activity in quartan malaria, the growing and older trophozoites of this species are, as a rule, more compact looking in thick films than are those of benign tertian. This is the commonest form of quartan malaria seen in blood examinations as the stage lasts for nearly two-thirds of the 72-hour cycle (*26*). The profuse, heavy, dark pigment scattered through the solid cytoplasm gives a dense appearance to a majority of the parasites. The picture often presented in the thick film by the quarter or third-grown trophozoites of quartan is so distinctive that when they are present in sufficient numbers, diagnosis is practically certain. In this stage the inconspicuous body of chromatin is often imbedded in a small, tight, rounded, heavily pigmented mass of cytoplasm. At first glance, one does not distinguish the characteristic red, blue, and brown of the parasite, but to the eye trained to pick them up, close examination will reveal the colors. Old trophozoites of quartan are also often quite compact and dark. They are larger than the ones mentioned above, and hence show more morphology. They often display quite nicely the elongated or streaky chromatin and peripheral arrangement of pigment, so characteristic of this stage in the thin film. Band forms of quartan cannot be certainly identified in thick films except perhaps in the thin edge.

Schizonts (presegmenting)

The presegmenting or immature schizonts of all the species of malaria have much the same appearance as in the thin film, except that there is a tendency toward more compactness. There is division of chromatin material, and the fewer of these divisions or the further from complete segmentation the parasite is, the more irregular in appearance the chromatin masses usually are. These chromatin masses often appear quite dark, reddish-purple and are sometimes (in

the thicker portion of the smear) distinguished with difficulty within the heavy cytoplasm. As the parasite approaches complete segmentation, the nuclear masses appear more regular in shape and the cytoplasm in the process of dividing may appear somewhat paler and in light wisps, particularly in benign tertian. The pigment is gradually collected into fewer and fewer groups as segmentation progresses in benign tertian but it has a tendency to concentrate late in quartan. The presegmenting schizonts are probably the ones most often passed over in the thick film by the inexperienced worker. The presegmenting forms of tertian and quartan malaria are (aside from infrequent ring forms) the most difficult stage in which to differentiate species in the thick film. However, these stages will usually be found accompanied by more readily recognized stages on which diagnosis may be based. There are times when the presegmenting forms of benign tertian are definitely larger than those of quartan, but one cannot depend completely on size.

The presegmenting stage of falciparum, when found in the peripheral blood, is as a rule smaller even than quartan and very compact. The best differentiating character is the small, rather prominent dark mass of pigment. Then, too, this form, when found is associated always with many young ring forms.

Mature schizonts (segmented)

Segmented schizonts of the three species resemble very closely the same stages in the thin film, practically the only difference being the absence of the cell outline and a possible shrinkage of the parasite. The individual merozoites often stand out from the group, each rounded mass of chromatin having its completely differentiated division or light blue zone of cytoplasm. The pigment at this stage is usually clumped in the center or near the edge of the parasite. Species may be determined by the comparative size of the rounded or oval cluster of merozoites and by the difference in their number. Benign tertian segmenters are not as frequently found as are other stages of this parasite. The quartan segmenter is the stage by which the inexperienced can diagnose this species most readily in the thick film. It rather regularly has about 8 merozoites—clearly separated. Frequently only the chromatin divisions and pigment show—with no sign of cytoplasm. It is unusual to find the segmenting schizont of falciparum except in severe cases, but in this species the merozoites are smaller than in quartan, more numerous than in the other species, and there is a small, dark, closely knit mass of pigment.

Gametocytes

In the thick film it is impossible to distinguish definitely the macrogametocytes of either benign tertian or quartan from the rounded or oval, mature trophozoites with a single mass of chromatin, rather

regular, dense cytoplasm and evenly dispersed pigment. Mature microgametocytes of these two species are more easily determined because of their very large, usually rounded or stellate nucleus, surrounded by a small amount of light staining or colorless cytoplasm containing numerous grains of prominent pigment. This nucleus often stains more deeply, comparatively speaking, than in the thin films. The parasite is often just a blob of chromatin with a halo of pigment granules. There is no other stage that resembles it closely. Occassionally, in thick films, exflagellation of fully-matured microgametocytes takes place on the slide before it dries.

The mature gametocytes of falciparum (crescents) are easily determined, as long as they retain their characteristic elongate or sausage-like shape, though it is often impossible in thick films to differentiate the sexes. In heavier portions of thick films, particularly when the blood dries slowly, mature aestivo-autumnal gametocytes assume a rounded shape, a change that would take place normally in the mosquito during the early stage of maturation. These forms may be confused with quartan parasites but the stages with which they are associated in the blood will aid in diagnosis as will certain characteristics of the form. The pigment will be in distinct rodlets and will often be arranged compactly with a clear halo or fringe of blue cytoplasm. Frequently, also, there is found projecting from one portion of the mass, a flag or tongue of pinkish or reddish staining material. The nature of this material is not known. It might possibly be the cell wall which contained the parasite; it might be extruded chromatin; but, at any rate, it is found sometimes in thick films with normally shaped crescents as well as with the rounded or "balled-up" forms. Immature crescents often take an elongated form with sharply pointed ends and with pigment scattered throughout their length. Usually typical crescents will be found in the thinner edges of the films, so that species identification is not dependent on the uncertain forms.

Sources of Confusion or Error

The inexperienced microscopist may be confused in the examination of thick films by bacteria or dirt from the skin; by dust particles on the slide; by vegetable spores, yeast cells or fungi from the air; and by bacteria, molds, protozoa, or other contaminants from the distilled water used in staining. Probably the chief source of dirt on the thick film is from the improperly cleaned finger. If the glass slide is very clean, the skin is cleaned thoroughly before taking the blood, the slide is not allowed to touch the skin, and the blood is dried protected from insects and dust; if utensils used for water and for staining are kept clean, and if the water used in staining and washing is kept free from growth and contamination, these disturbing factors will be entirely eliminated or reduced to a negligible mini-

mum. Artefacts which may deceive the inexperienced will be found frequently to lie above the blood plane, or they may be refractile and focus out of the field unevenly. Sometimes red dots appear in the preparation without any visible cytoplasm. It is possible that some of these are at times remnants of malaria parasites, but *one should never call a slide positive on these dots alone.* The dots may be staphylococci or other cocci or they may be products of degeneration of the red cells. If the dots are evenly distributed throughout the smear they are more likely to be associated with the cells, than if they are found in clumps or only in a part of the smear. If a red stained coccus from the skin or small nuclear remnant lies adjacent to a blue stained particle of cellular substance or fibrin, there may be a resemblance to a parasite. This rarely occurs more than once or twice on a single slide and numbers of free cocci will usually be found also, whereas, in well-stained smears free chromatin dots are rarely found. As one becomes more familiar with the thick film one is less likely to mistake any foreign body for a parasite. According to Barber and Komp a good general rule is not to consider anything a parasite which may be interpreted as an artefact. Thick films should not be diagnosed as positive on what appears to be only one parasite. The parasite should be unmistakable, or search should be continued until others are found. If this is impossible later smears should be made.

V. HISTORY OF STAINING AND OF THICK FILM TECHNIQUE

The demonstration of malaria parasites in the blood is one of the most important phases of blood staining and any description or history (*27*) of the work done on the former is of necessity closely knit with the latter. Erhlich (1879) was the first person to recognize the difference between acid and basic dyes and to appreciate the significance of that difference in staining blood. In 1891, Romanowsky described a technique for the use of a methylene blue-eosin combination. By this technique he was the first to obtain the blood picture which we consider typical today, and also the first to demonstrate the nucleus of the malaria parasite in the blood, thus stimulating interest in such stains. Romanowsky had obtained his results without knowing the underlying principles of the stain; consequently the technique did not always work consistently.

Important contributions toward a satisfactory stain were made by Uma (1891), Nocht (1898), Jenner (1899), Reuter and Leishman (1901) (*28*) and others. Michaelis (1901) basing his experiments on the chemical work of Berthsen (1885), was the first to understand that the "red from methylene blue" of Nocht or methylene azure was the staining element in the combined eosin-methylene blue which gave the coveted chromatin stain. Though it was several years after this before Kehrmann and Berthsen obtained a correct understanding of

the chemistry of methylene azure, Giemsa in 1902 began putting Michaelis' principles to practical use and obtained a very satisfactory parasite stain made from the eosinate of Azure II which is a mixture of equal parts of methylene blue and methylene azure (Azure I) together with a small additional amount of Azure II. He succeeded in producing a ready-made, stable mixture in glycerine and methyl alcohol which was diluted before use. It was in 1904 that Grübler put Giemsa's stain on the market.

It is a coincidence that the thick film technique, now recognized as the surest method for diagnosis of malaria, and Giemsa's stain, the most satisfactory used yet with this method, should have come into use at so nearly the same time, though it was a number of years before the two were used together to any great extent.

While Sir Ronald Ross first had the idea for the thick film as far back as 1895, it was not until 1902, in a lecture before the Institute of Public Health in London, that he first described his method for the more rapid detection of Plasmodia in the blood. It was published shortly thereafter (*6*). His method was to dissolve the hemoglobin from a fairly thick film of blood (dried without fixation) by covering it with an aqueous eosin solution for about 15 minutes. He then washed it and stained it with methylene blue. He adjudged this method to be 25 times better than the ordinary thin preparation for diagnostic purposes. Ruge (1903), thinking that the thick films were much too easily separated from the slide in staining, modified the method, combining dehemoglobinization with fixation by using ether and alcohol. Later, he used formalin and acetic acid on the dried smear before staining with dilute Giemsa (1 drop to 1 cc. for 20 or 30 minutes). When the German Sleeping Sickness Expedition (1907) headed by Koch did its work in East Africa, the members adopted the thick film method (*21*), and found that the water of diluted Giemsa stain is quite sufficient to dehemoglobinize simultaneously with the staining process.

In 1911, William M. James (7) described a modification of the staining method which received his name. He used an acid alcohol (about 1% HCl in ethyl alcohol) for fixation and dehemoglobinization, washed the slide, dried it, and stained it flat with one of the ordinary blood stains for a longer period of time than with the thin films. He gave as his objection to Ross' original method the facts that the nucleus of the parasite did not stain, and part of the blood was lost from the slide in staining. The Romanowsky stain and the fixation were intended to correct these objections.

The Field Investigations of Malaria Section of the Public Health Service was organized in 1912 under the direction of R. H. von Ezdorf. Almost immediately malaria surveys were begun in North Carolina and Arkansas, and the James method of staining was at first used,

with some variation in the length of time of dehemoglobinization and fixation, and of washing out the acid afterwards. Taylor, one of von Ezdorf's assistants (*10*), tells of the use of a modification of Giemsa stain instead of Wright's or Leishman's, or another Romanowsky stain, and tells also of putting the slides in the diluted stain rather than pouring the stain on the slide. After four years' work with the thick film method Bruce Mayne published (*29*) the method which he had found most satisfactory. This was based upon the James' method, but the staining was done by steps—polychrome methylene blue, then eosin, then the methylene blue again.

In 1920 S. P. James (*30*) gave his modification of the thick film technique, the dehemoglobinization of thick films by standing them in tap water for 10 minutes, then staining them without drying or fixing with Leishman or Giemsa stain in a glass staining jar. He claimed that the method was crude and admitted of errors, that it was not applicable to field work in the open and that it should be used only to supplement the thin film.

In spite of the work of all these men there was a need for improvement in the quality of the preparations as generally made, and simplification of the method of handling large numbers of slides under field conditions. In 1924 Barber and Komp (*9*) presented to the International Conference on Health Problems in Tropical America their modification of the thick film method of examination, with a view of meeting the above mentioned needs. Their insistence upon clean slides and avoidance of dust, etc., in the smears was intended to correct the loss of blood smears in staining. For staining they used practically the modification used by the German Sleeping Sickness Commission—simultaneous dehemoglobinization and staining in dilute Giemsa. The method they used for processing large numbers of smears at one time was ingenious and workable as a field survey method and did a great deal toward popularizing the thick film. In 1929 they revised and expanded their article making some changes based on their experience during the intervening time (*22*). In 1933 Komp wrote, "Additional Notes on the Preparation and Examination of Thick Films for Malaria Diagnosis" (*31*), which contained a number of valuable points on technique not brought out in the previous paper.

Unsettled conditions in Europe in 1939 stimulated a study of American dyes with a view of producing stains consistently satisfactory on malaria parasites, particularly in the thick film. In this study it was found that Azure B, not Azure A, is the principal ingredient of German Azure I, (the significant part of the Giemsa formula). With this knowledge as a basis, formulae were devised for the compounding of satisfactory Giemsa solutions (formula "A" below) (*32*). To make these entirely reliable, however, it was found that Azure B

should be added to the list of certified dyes, with its dye content determined. In the absence of certified Azure B, and in order to facilitate the manufacture of Giemsa stain from American dyes, another study was made to devise a Giemsa stain from the eosinates of Azure A, Azure B, and Methylene Blue, since the eosinates are easily made and are of uniform composition and dye content (Formula "M" below) (*33*).

Formula "A"				Formula "M"
	Nocht formula (cc)	Pure dye weight	Total dye weight	
	1:1,000 solution	*Mg.*	*Mg.*	
Azure B (80% est.)	2.0	200	250	Azure B eosinate 250 mg.
Azure A 90%	0.5	50	55	Azure A eosinate 50 mg.
Methylene blue 87%	2.7	270	310	Methylene blue eosinate 200 mg.
Eosin Y 93%	5.0	500	537	Methylene blue chloride (87%) 100 mg.
Glycerine		50 cc		Glycerine 50 cc.
Methyl alcohol		50 cc		Methyl alcohol 50 cc.
Total		100 cc stain		Total 100 cc stain.

Numbers of workers have written of their use of and satisfaction with the thick film method. J. Pratt Johnson in 1921 (*34*), in a report on his experience in the blood examination of 250,000 troops returned from a tropical campaign, stated that the thick film enormously increased the number of positive results and enhanced considerably the value of the blood examination. He took note also of the speed with which large numbers of slides can be handled.

In 1925 J. A. Sinton and A. C. Banerjea (*8*) made a plea for the more general adoption of the thick film method and gave proof of its superiority in diagnosis. Krauss in 1931 (*12*) urged greater interest in the proper diagnosis of malaria and tried to show physicians the value of the thick film examination in this relation.

The malaria investigations laboratory of the United States Public Health Service has been using the thick film technique for malaria surveys intermittently since 1913, on a full time basis with the present technique since 1928.

In 1933 cooperation of Federal relief agencies with the States on drainage projects for the control of malaria afforded an opportunity for a much needed blood index in selected localities in the southern United States. During the spring of 1934 a wide scale survey was taken. Many of the blood smears were examined by some of the respective State laboratories, but a total of around 145,000 thick blood films from twelve southern States (*38*) were stained and subsequently examined at the National Institute of Health by a group of twenty technicians. These technicians quickly learned to examine 50 slides daily, with an error of less than 2 percent. Except for the thick film method, and the fact that stained slides can be kept intact

for long periods, such an undertaking would have been highly unpractical.

Between the spring of 1932 and the fall of 1941, approximately fifty classes for technicians have been held in nine southern States. Students from six other State laboratories also attended these classes, which were usually sponsored by the respective State health departments. The course of study consisted of instruction in the recognition of malaria parasites as well as in the proper technique of taking, staining, and examining blood smears. The courses were usually of 2 weeks' duration. At least 500 students have participated in these classes, many for the full period and others for such time as could be spared from their regular duties. As a result of these classes at least six of the States use the thick film technique for much of their diagnostic work in malaria, as well as for malaria surveys. Among the other southern States, use of this technique is increasing.

In connection with the National Defense program and at the request of the War Department, this summer (1941) classes in thick film technique were conducted at the Fourth Corps Area laboratory at Fort McPherson, Ga. Technicians from camps and hospitals of the army in the southern States were detailed to attend.

The method of teaching thick film identification of malaria plasmodia herein described has been developed during the past 13 years, throughout which period chiefs of laboratories have submitted both positive and negative slides after examination by many of the students as a check on their work. The uniformity of excellence of slide preparation and the high degree of accuracy of species differentiation maintained by them is the reason for this presentation.

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PLATE I.—*P. vivax*

1. Normal sized red cell with marginal ring form trophozoite.
2. Young signet ring form trophozoite in a macrocyte.
3. Slightly older ring form trophozoite in red cell showing basophilic stippling.
4. Polychromatophilic red cell containing young tertian parasite with pseudopodia.
5. Ring form trophozoite showing pigment in cytoplasm, in an enlarged cell containing Schüffner's stippling.[1]
6, 7. Very tenuous medium trophozoite forms.
8. Three ameboid trophozoites with fused cytoplasm.
9, 11, 12, 13. Older ameboid trophozoites in process of development.
10. Two ameboid trophozoites in one cell.
14. Mature trophozoite.
15. Mature trophozoite with chromatin apparently in process of division.
16, 17, 18, 19. Schizonts showing progressive steps in division ("presegmenting schizonts").
20. Mature schizont.
21, 22. Developing gametocytes.
23. Mature microgametocyte.
24. Mature macrogametocyte.

[1] Schüffner's stippling does not appear in all cells containing the growing and older forms of *P. vivax* as would be indicated by these pictures, but it can be found with any stage from the fairly young ring form onward.

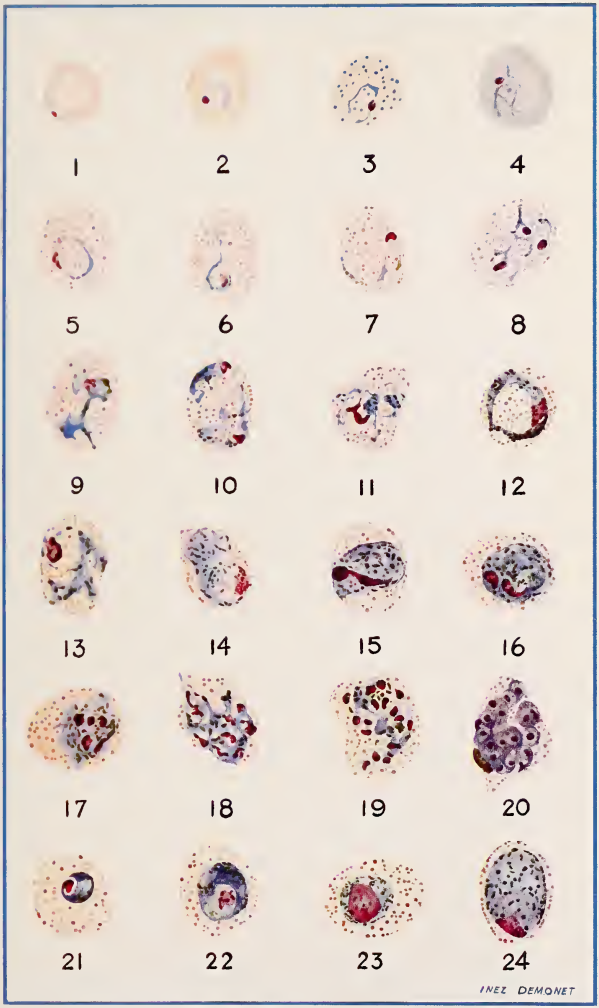
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PLATE II.—*P. malariae*

1. Young ring form trophozoite of quartan malaria.

2, 3, 4. Young trophozoite forms of the parasite showing gradual increase of chromatin and cytoplasm.

5. Developing ring form trophozoite showing pigment granule.

6. Early band form trophozoite—elongated chromatin, some pigment apparent.

7, 8, 9, 10, 11, 12. Some forms which the developing trophozoite of quartan may take.

13, 14. Mature trophozoites—one a band form.

15, 16, 17, 18, 19. Phases in the development of the schizont ("presegmenting schizonts").

20. Mature schizont.

21. Immature microgametocyte.

22. Immature macrogametocyte.

23. Mature microgametocyte.

24. Mature macrogametocyte.

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PLATE III.—*P. falciparum*

1. Very young ring form trophozoite.

2. Double infection of single cell with young trophozoites, one a "marginal form," the other "signet ring" form.

3, 4. Young trophozoites showing double chromatin dots.

5, 6, 7. Developing trophozoite forms.

8. Three medium trophozoites in one cell.

9. Trophozoite showing pigment, in a cell containing Maurer's spots.

10, 11. Two trophozoites in each of two cells, showing variation of forms which parasites may assume.

12. Almost mature trophozoite showing haze of pigment throughout cytoplasm. Maurer's spots in the cell.

13. Aestivo-autumnal "slender forms."

14. Mature trophozoite, showing clumped pigment.

15. Parasite in the process of initial chromatin division.

16, 17, 18, 19. Various phases of the development of the schizont ("presegmenting schizonts").

20. Mature schizont.

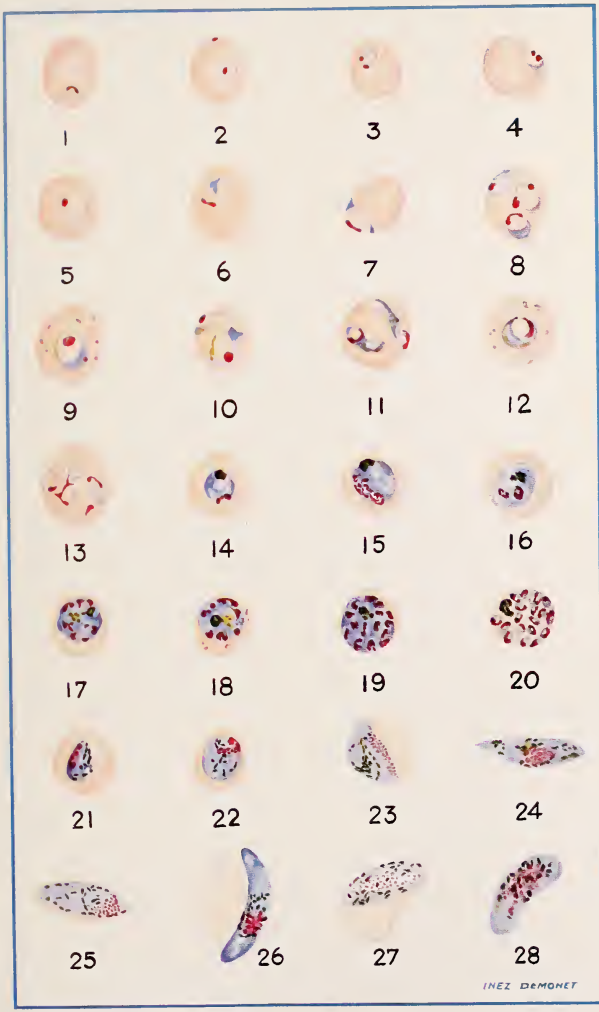
21, 22, 23, 24. Successive forms in the development of the gametocyte—usually not found in the peripheral circulation.

25. Immature macrogametocyte.

26. Mature macrogametocyte.

27. Immature microgametocyte.

28. Mature microgametocyte.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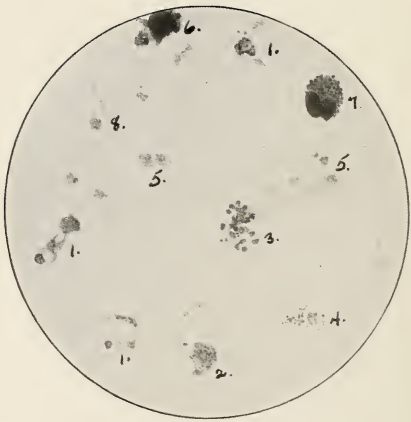


PLATE IV.—*P. vivax*—thick film

1. Ameboid trophozoites.
2. Schizont—2 divisions of chromatin.
3. Mature schizont.
4. Microgametocyte.
5. Blood platelets.
6. Nucleus of neutrophil.
7. Eosinophil.
8. Blood platelet associated with cellular remains of young erythrocytes.

Plate IV.

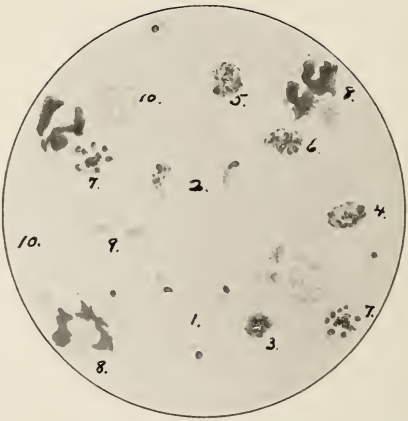
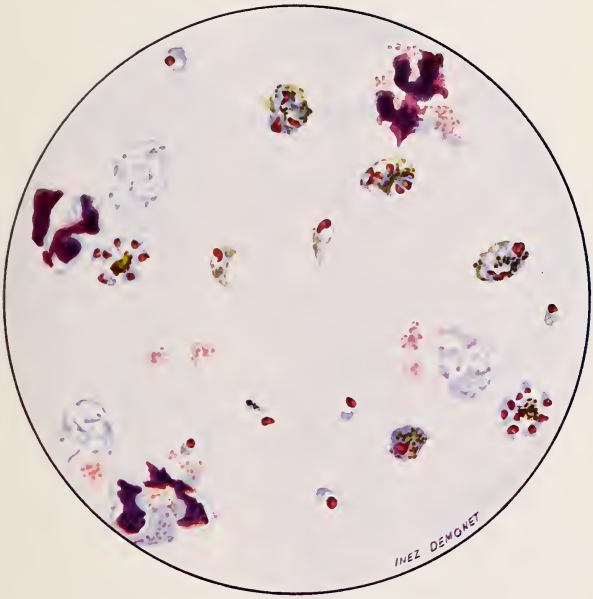


PLATE V.—*P. malariae*—thick film

1. Small trophozoites.
2. Growing trophozoites.
3. Mature trophozoites.
4, 5, 6. Schizonts (presegmenting) with varying numbers of divisions of the chromatin.
7. Mature schizonts.
8. Nucleus of leucocyte.
9. Blood platelets.
10. Cellular remains of young erythrocytes.

PLATE V.



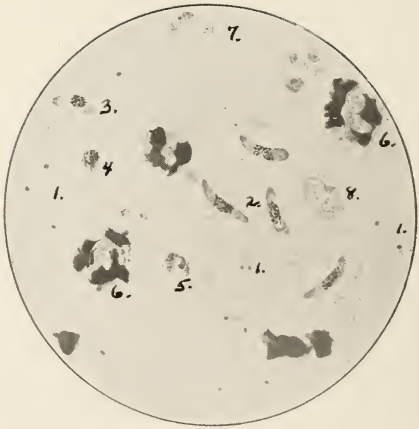
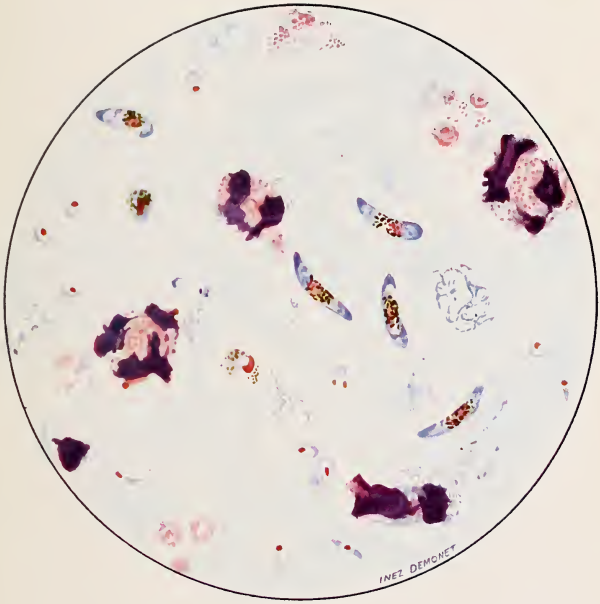


PLATE VI.—*P. falciparum*—thick film

1. Small trophozoites.
2. Gametocytes—normal.
3. Slightly distorted gametocyte.
4. "Rounded-up" gametocyte.
5. Disintegrated gametocyte.
6. Nucleus of leucocyte.
7. Blood platelets.
8. Cellular remains of young erythrocyte.

PLATE VI.



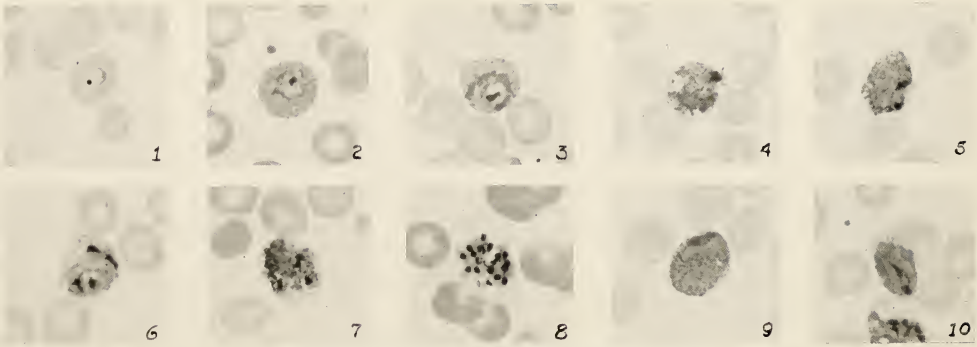


PLATE VII.—Photographic reproductions of stages of *P. vivax*

1. Young trophozoite.
2. Trophozoite of moderate size. Enlargement of cell and Schüffner's stippling evident.
3. Still older trophozoite showing pseudopodial process.
4. Mature trophozoite with irregular outline.
5. Schizont showing initial division of chromatin.
6. Older schizont showing four divisions of chromatin.
7. Still older schizont.
8. Mature schizont with about 16 divisions of chromatin and some clumped pigment.
9. Macrogametocyte.
10. Microgametocyte near a presegmenting schizont.

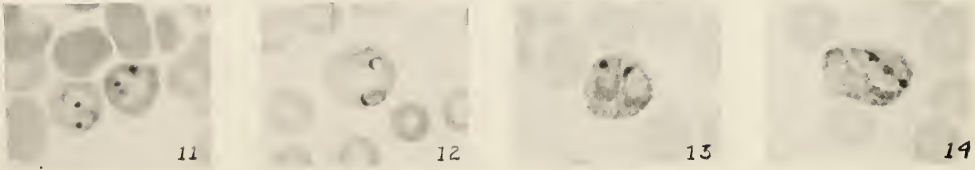


PLATE VIII.—Cells showing double infection of *P. vivax*

11. Two cells each containing two young trophozoites.
12. Enlarged cell containing two slightly ameboid young trophozoites.
13. Schüffner's stippling quite apparent in cell infected with two medium grown trophozoites.
14. Double infection of a cell by a presegmenting schizont and another old parasite—apparently a macrogametocyte.

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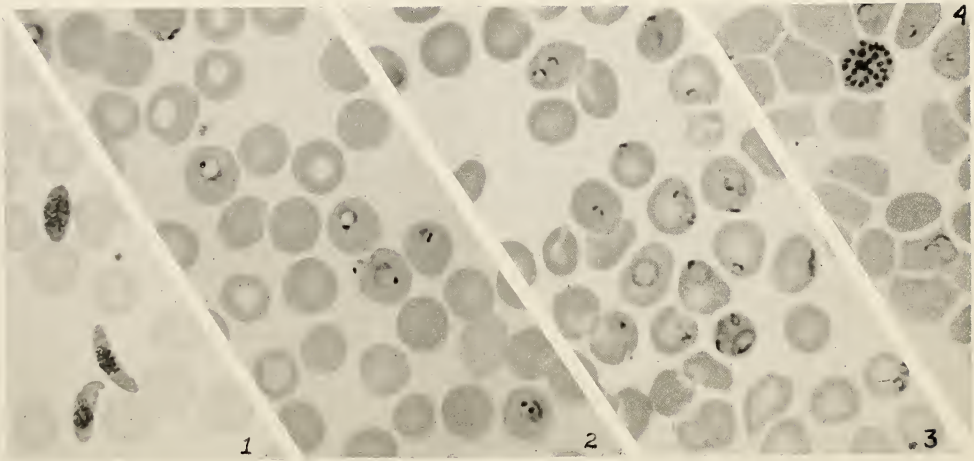


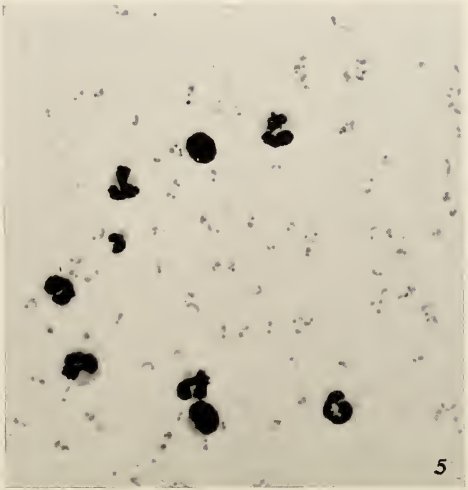
PLATE IX.—Photographic reproductions of stages of *P. falciparum*

1. Microgametocyte at top, two macrogametocytes lower down.

2. Heavy infection of ring form trophozoites often found in fresh infections of aestivo-autumnal malaria, showing double chromatin dots, double infection of the cell, marginal forms and signet ring forms.

3. Heavy infection from moribund case of aestivo-autumnal malaria—note variation in size of trophozoites and number of multiply infected cells.

4. Mature schizont of aestivo-autumnal from the same case as number 3.



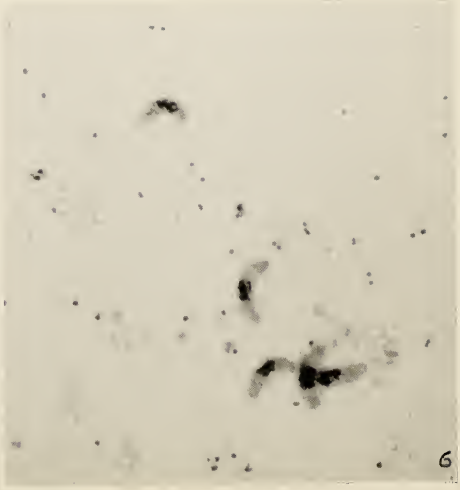


PLATE X.—Thick films of *P. falciparum*

5. Heavy infection, ring-stage trophozoites.
6. Young trophozoites and gametocytes. Background clouded with remains of young red cells.

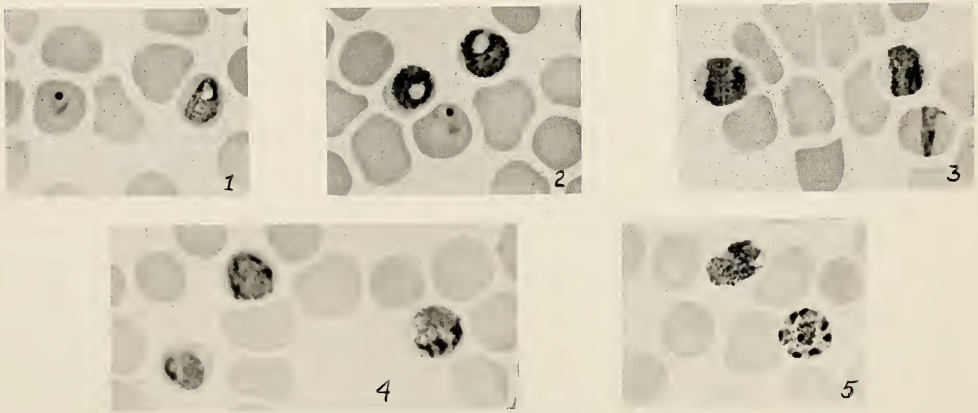


PLATE XI.—Photographic reproductions of stages of *P. malariae*

1. Young ring form trophozoite and medium grown trophozoite.
2. One ring form with slight pseudopodial process and two older trophozoites each with elongated chromatin mass and profuse, heavy pigment.
3. The band form parasites, the one on the left showing two divisions of chromatin, the other two being different stages of trophozoites.
4. Reading from the left: medium grown trophozoites; two presegmenting schizonts—one with four, the other with three, divisions of chromatin.
5. A large trophozoite and a mature schizont.

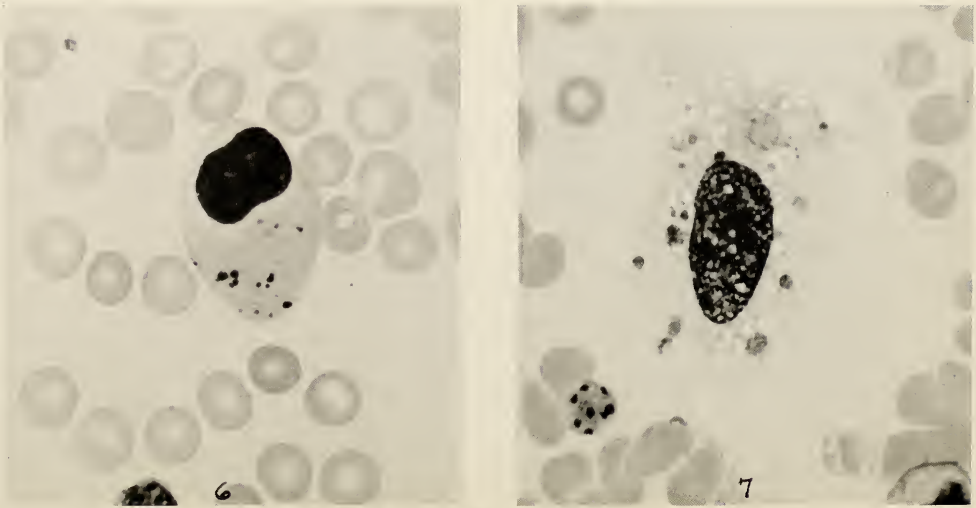


PLATE XII.—Phagocytized pigment.

6. Monocyte containing malarial pigment.
7. Endothelial cell from severe case of malaria, showing a great many masses of malarial pigment.

PLATE XIII.—Making thick blood smears

1. Cleaning finger with alcohol.
2. Making the prick.
3. Making thick smear.
4. Drying laboratory slide in Petri dish.
5. Showing inverted "T" block supporting two slide boxes for blood surveys. Open box contains slides on which there are thick and thin smears.